

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**



Express Mail No.: EL 500 576 074 US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

RECEIVED

SEP 25 2002

TECH CENTER 1600/2900

Application of: Spear *et al.*

Confirmation No.: 3399

Application No.: 09/924,231

Group Art Unit: 1648

Filed: August 8, 2001

Examiner: Wortman, D.

For: PHARMACEUTICAL COMPOSITIONS
COMPRISING HERPES VIRUS ENTRY
RECEPTOR PROTEIN

Attorney Docket No.: 7853-239

DECLARATION OF ABBIE CELNIKER UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Abbie Celniker do declare and state:

1. I presently hold the position of Vice President of Biotherapeutics at Millennium Pharmaceuticals, Inc. Millennium Pharmaceuticals, Inc. is the exclusive licensee of the above-identified application ("the '231 application"). My academic and technical experience and honors, and a list of my publications are set forth in my *curriculum vitae*, attached as Exhibit 1.

2. I have read and am familiar with the following materials:
- the '231 application;
 - the claims currently pending in the '231 application;
 - the Office Action, dated March 5, 2002, issued in connection with the '231 application; and
 - Exhibits 2-9 discussed below.

3. The invention claimed in the '231 application is directed to pharmaceutical compositions comprising Herpes Virus Entry Mediator ("HVEM") protein. HVEM is also

known in the art as TR2 (TNF Receptor-, or TNFR-, related 2), ATAR (another TRAF (TNF Receptor Associated Factor)-associated receptor), and HveA (herpesvirus entry protein A).

4. HVEM is a member of the family of receptors designated the tumor necrosis factor receptor/nerve growth factor receptor (TNFR/NGFR) family ('231 specification at page 1, last paragraph). HVEM was identified by the present inventors on the basis of its ability to confer susceptibility to herpes simplex virus type 1 ("HSV1") upon Chinese Hamster Ovary ("CHO") cells ('231 specification at pages 10-12). Briefly, as described by the specification, CHO cells that do not express HVEM are resistant to HSV1 entry, as evidenced by lack of expression of β -galactosidase, a reporter gene carried by the virus employed in the studies described in the '231 specification, following contacting the cells with the virus. In contrast, CHO cells that recombinantly express HVEM are sensitive to HSV1 infection, which is evidenced by their expression of the viral reporter β -galactosidase.

5. The references attached hereto as Exhibits 3-6, published before what I understand is the filing date of the first parent application of the '231 application and discussed in more detail in ¶¶ 6-9 below, corroborate that one of ordinary skill in the art would recognize that a dominant negative receptor of the TNF receptor family, such as a soluble receptor, interferes with binding of a TNF-class ligand to its receptor. These references further corroborate that one of ordinary skill in art would recognize that, where a TNF-class ligand mediates entry of a pathogen into a host cell, such as a human cell, via a TNF receptor family member, a soluble form of the receptor would interfere binding of the ligand to the host cells, and therefore inhibit entry of the pathogen into the host cell. Accordingly, one of ordinary skill in the art would recognize that a dominant negative form of HVEM, such as soluble HVEM, of which the native form (*i.e.*, cellular membrane-bound form) was shown by the inventors to mediate entry of herpes virus into mammalian cells, would be useful in inhibiting herpes virus entry into mammalian cells. Thus, one of ordinary skill in the art at the time the earliest parent of the '231 application was filed would recognize that an exogenous HVEM molecule which sequesters herpes virus from binding to cellular HVEM and thus interferes with the ability of herpes virus to infect cells could be formulated into a pharmaceutical composition for therapeutic or prophylactic use against herpes virus.

For these reasons and the reasons discussed in ¶¶6-16 below, one of skill in the art would recognize that dominant exogenous HVEM molecules, such as soluble HVEM, can be used to treat or prevent infections by HSV. Further, one of skill in the art, using the teachings of the specification and routine methodology, would be able to make and administer pharmaceutical compositions comprising such HVEM molecules. In particular, pharmaceutical formulations are taught in the specification of the '231 application, for example in Section V at pages 24-25, and are also well known in the art (see, for example, Remington's Pharmaceutical Sciences, Alfonso R. Gennaro ed., Mack Publishing Co. Easton, Pa., 19th ed., 1995, pp. 1495-1560, attached hereto as Exhibit 2). Methods of administration are also both taught in the specification (second paragraph on page 25) and well known to the skilled artisan.

**ADMINISTRATION OF HVEM IS EXPECTED TO INHIBIT
INFECTION BY OR SPREADING OF HERPES VIRUS**

6. Gray *et al.* ("Gray"), Exhibit 3 attached hereto, establishes that a soluble receptor of the TNF receptor family inhibits the biological activity of its ligand. Gray demonstrates that conditioned medium from COS cells transfected with the extracellular domain of the TNF receptor both inhibited the binding of TNF α to cells which express the endogenous, *i.e.*, membrane-bound, receptor by approximately 70% (Fig. 4A at page 7383) and inhibited a biological activity of TNF α , induction of cytolysis of WEHI cells, by about 60% (Fig. 4B at page 7383).

7. Ashkenazi *et al.*, Exhibit 4 attached hereto ("Ashkenazi"), demonstrates that a soluble form of a TNF receptor, namely a fusion protein of the extracellular domain of human type 1 TNF receptor and an immunoglobulin ("TNFR-IgG"), inhibits both *in vivo* as well as *in vitro* biological activity of TNF. *In vitro*, TNFR-Ig was able to block TNF-induced lysis of actinomycin-treated murine cells (Fig. 3 at page 10537). Further, *in vivo*, administration of TNFR-Ig to experimental mice was able to protect the mice against TNF-mediated toxic shock induced by *Salmonella*-derived endotoxin (Fig. 4 at page 10538). Accordingly, Ashkenazi establishes that administration of a soluble form of a TNF class of receptor to a mammal inhibits the biological activity of the endogenous receptor.

8. Ward *et al.*, Exhibit 5 attached hereto ("Ward"), describes using a soluble form of CD4, the human immunodeficiency virus ("HIV") entry receptor, comprising two extracellular immunoglobulin domains of CD4 and a human immunoglobulin, referred to hereinafter as CD4-Ig, to block HIV-1 infection in chimpanzees. A comparison was made of the course of HIV infection in chimpanzees treated with CD4-Ig (Animals 37 and 43) prior to and following challenging the animals with HIV versus a control animal that received the HIV challenge but no CD4-Ig treatment (Animal 62). Although the control chimpanzee, Animal 62, showed signs of HIV infection as early as three weeks following the HIV challenge, the two chimpanzees that received the CD4-Ig regimen were HIV negative by three different assays at the end of the 47-week testing period (Table 1 at page 435).

9. Greve *et al.*, attached hereto as Exhibit 6 ("Greve"), describes experiments in which the effect of two soluble forms of intercellular adhesion molecule-1 ("ICAM-1"), the receptor of the majority of human rhinoviruses, on the binding of human rhinovirus type 3 (HRV3) to full length ICAM-1 and HRV3 infectivity. One of the soluble forms of ICAM-1 tested in this study corresponded to the entire extracellular domain of ICAM-1, and the other to ICAM-1's two N-terminal immunoglobulin-like domains, referred to hereinafter as tICAM(453) and tICAM(185), respectively. In an *in vitro* binding assay, both soluble forms of ICAM-1 were capable of inhibiting the binding of radiolabeled HRV3 to ICAM-1 immobilized on a microtiter dish (Fig. 3A at page 6018). tICAM(453) and tICAM(185) were also capable of inhibiting HRV3 infection of HeLa cells (Fig. 3B at page 6018), and further inhibited HRV3 replication in HRV3- infected HeLa cells (Fig. 3B at page 6018).

10. I conclude, based on the references described in ¶¶ 6-9 above and the data described in the '231 specification, that recombinant HVEM would bind to and sequester HSV1 particles, thereby preventing their binding to cellular HVEM or any other cellular receptor, thus preventing HSV1 infection of cells or the spread of HSV1 from cell to cell following infection.

SOLUBLE HVEM IS EXPECTED TO BE USEFUL IN TREATING OR PREVENTING HSV INFECTIONS

11. Wild-type HSV-1 can use HVEM for cellular entry, as transfection of CHO cells with HVEM renders them susceptible to HSV-1 ('231 specification, page 13 at second paragraph). Viral entry through HVEM is thought to be at least in part mediated by HSV viral envelope glycoprotein D, or gD ('231 specification, page 13 at third paragraph).

12. A phenomenon called gD-interference has been observed for herpes viruses. Briefly, gD interference refers to cellular resistance to herpesvirus infections in cells in which wild-type gD is expressed. The inventors of the '231 application observed gD interference of HSV1 infections in gD- and HVEM-expressing CHO cells, even when the gD was of HSV2 origin ('231 specification at pages 13-14). Because gD interference is thought to occur by gD sequestration of a cellular receptor, the gD interference observed by the inventors suggests that HVEM interacts with gD of both HSV1 and HSV2. This is consistent with the hypothesis that gD interference results from competition between cell-associated gD and virion-associated gD for a common target, *i.e.*, HVEM. This observation has been verified (see, *e.g.*, Montgomery *et al.*, Exhibit 7 attached hereto, and Whitbeck *et al.*, 1997, Exhibit 8 attached hereto).

13. Although HVEM can mediate cellular entry of HSV1 and HSV2 in CHO cells, HVEM-expressing CHO cells remain resistant to certain mutant strains of HSV1 ('231 specification, page 13 at third paragraph), suggesting the presence of other receptors. Indeed, other receptors for herpesviruses have been identified following the effective filing date of the '231 application (see, *e.g.*, background section of Whitbeck *et al.*, 1999, Exhibit 9 attached hereto).

14. However, despite the existence of more than one cellular herpesvirus receptor, evidence suggests that one particular region of herpesvirus gD protein is responsible for binding to a number of these receptors (see, *e.g.*, Whitbeck *et al.*, Exhibit 9, in particular Fig. 11 at page 9888).

15. Because herpesvirus gD protein is capable of binding more than one cellular receptor through a common domain, one of skill in the art would conclude that obstruction of

this region in gD would inhibit gD from binding to its cellular receptors and therefore inhibit viral infection of cells, in a manner analogous to gD interference among various strains of HSV. This is consistent with the observation that the region of gD responsible for binding to HVEM and another cellular receptor, HveC, overlaps a region of gD that is recognized by a particular class of herpesvirus neutralizing antibodies (*see, e.g., Whitbeck et al., Exhibit 9, in particular Fig. 11 at page 9888*).

16. In view of the above observations, one of skill in the art would expect that soluble HVEM, given its ability to bind to gD of wild type HSV1 and HSV2, would be useful in preventing the binding of HSV1 and HSV2 to a cellular receptor, thereby inhibiting infection and spreading of these viruses in mammalian cells.

17. In summary, the teaching presented in the '231 application, coupled with the state of the art at the time the first parent of the '231 application was filed, would allow one of skill in the art to routinely make and use pharmaceutical compositions comprising soluble HVEM protein. One of skill in the art would expect administration of such compositions to be useful in achieving clinically beneficial results in the treatment or prevention of infections by wild type HSV1 and HSV2.

18. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated:

13 AUG 2002



Abbie Celniker

Attachments:

- Exhibit 1: *Curriculum Vitae* of Abbie Celniker.
- Exhibit 2: Remington's Pharmaceutical Sciences, Alfonso R. Gennaro ed., Mack Publishing Co. Easton, Pa., 19th ed., 1995, pp. 1495 to 1560.
- Exhibit 3: Gray *et al.*, 1990, "Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein," Proc. Nat'l Acad. Sci. U.S.A. 87:7380-7384.
- Exhibit 4: Ashkenazi *et al.*, 1991, "Protection against endotoxic shock by a tumor necrosis factor receptor immunoadhesin," Proc Natl Acad Sci U.S.A. 88(23):10535-39.
- Exhibit 5: Ward *et al.*, 1991, "Prevention of HIV-1 IIIB Infection in Chimpanzees by CD4 Immunoadhesin," Nature 352:434-436.
- Exhibit 6: Greve *et al.*, 1991, "Mechanisms of Receptor-Mediated Rhinovirus Neutralization Defined by Two Soluble Forms of ICAM-1," Journal of Virology 65(11):6015-6023.
- Exhibit 7: Montgomery *et al.*, 1996, "Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family," Cell 87:427-436.
- Exhibit 8: Whitbeck *et al.*, 1997, "Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the tumor necrosis factor receptor superfamily and a mediator of HSV entry," J. Virol. 71(8):6083-93.
- Exhibit 9: Whitbeck *et al.*, 1999, "The major neutralizing antigenic site on herpes simplex virus glycoprotein D overlaps a receptor-binding domain," J. Virol. 73(12):9879-90.

Abbie C. Celniker, Ph.D.
560 Chestnut Street
Newton, MA 02468
Home: 617-332-0067 Fax: 617-969-7030
Acelniker@AOL.com

Education:

1986, Ph.D., Molecular Biology, University of Arizona. Immunological Studies of Human Cathepsin D

1980, B.A., Biology, University of California, San Diego

Experience Overview:

- Extensive experience in the area of recombinant protein pharmaceutical development and commercialization including; functional area oversight, regulatory submissions, interactions with CEBR and CDER, and project team leadership and participation.
- Scientific expertise in the areas of transplantation biology, co-stimulation, growth and wasting.
- Technical expertise in the areas of; monoclonal antibody development and characterization, immunoassay and analytical methods development and Preclinical biology.
- Managerial experience including the management of individuals and groups consisting of Ph.D., DVM, Post-doctoral and research associate level positions.
- Compliance experience including the organization and maintenance of GLP and GMP compliant laboratories and information management systems.
- Translational biology experience focused on the integration of novel analytical methods into clinical studies and the movement of therapeutic proteins from research into the clinic.

Employment:

June 2000 to Present, Vice President, Biotherapeutics, Millennium Pharmaceuticals, Cambridge, MA.
Responsibilities include:

- Oversight of the following functional areas: Therapeutic Antibody Technology Group, Protein Sciences (discovery and process development), Biological Assay Development, Mouse Models Development and the Animal Resources Group.
- Participation on the Discovery Scientific Review Committee, Development Scientific Review Committee and Product Team (development portfolio management)

October 1999 to June 2000, Assistant Vice President, Predevelopment – Biopharmaceutical Core Technologies, **Genetics Institute of Wyeth Ayerst Research**, Andover/Cambridge, MA.

Responsibilities included:

- Oversight of the following functional areas: Therapeutic Antibody Technology Group, Research Protein Supply, Proteomics, Bioanalytical Sciences, Pharmacokinetic and Pharmacodynamic Sciences, Laboratory Animal Resources, Preclinical Scientific Communications, Research Operations and the External Research Department.
- Oversight of “predevelopment process” for therapeutic proteins moving from discovery research into development (Lead Candidate through IND).
- Preclinical Project Team Leader for the Anti-B7.1/Anti-B7.2 Program in GvHD and Renal Transplantation

November 1993 to June 1999, Director /Senior Scientist of Bioanalytical Sciences at **Genetics Institute**, Andover, MA. Responsibilities included:

- Oversight of the Antibody Technology Group, Bioanalytical Sciences and the Preclinical Transcriptional Profiling group (Gene Expression Monitoring).
- The establishment and oversight of a GLP compliant immunoassay lab, including laboratory automation (sample tracking, sample manipulation and data transfer), assay validation and facility management.
- Member of the Analytical Coordinating Group (ACG) responsible for the immunoassays used for identity testing, ligand binding analysis and immunoassays for host cell protein impurities to support process and product development.
- Oversight of the assessment and interpretation of anti-product immune responses for Preclinical and clinical studies.
- Preclinical Project Team Leader for the Anti-B7.1/Anti-B7.2 Program in GvHD and Renal Transplantation

May of 1993 to November 1993, Associate Director/Senior Scientist, Medicinal and Analytical Chemistry, **Genentech Inc.**, South San Francisco, CA. Responsibilities included:

- Oversight of the Bioanalytical Methods Development group, responsible for immunoassay development for research, Preclinical, clinical and product development support
- Preclinical Research Project Team Leader for the IGF-1 Program

June 1986 to May of 1993, Scientist, Medicinal and Analytical Chemistry, **Genentech Inc.**, South San Francisco, CA. Responsibilities included:

- Development of antibodies and immunoassays for the quantitation of human and animal growth hormones in serum and urine and the assessment of the anti-growth hormone antibody response.
- Development of antibodies and immunoassays for the quantitation of human Insulin-like Growth Factor 1 (IGF-1) and IGF-1 binding proteins in serum and urine to support preclinical and clinical pharmacokinetics and pharmacodynamics
- Development of antibodies and immunoassays for the quantitation of gamma interferon, TNF-alpha, HSA, Human Relaxin, Pro-Relaxin, and Relaxin "A" and "B" chains in serum, urine and cell expression systems
- Development of immunoassays for the quantitation of E. coli and CHO derived host cell protein impurities

1984 to 1986, Research Associate, **University of Arizona Cancer Center**, Veteran's Administration Hospital, Tucson, AZ. Responsibilities included:

- Establishment of primary cells lines from prostate tumor and benign prostatic hypertrophy specimens.
- Development of assays to differentiate cytostatic from cytotoxic biological response modifiers.
- Development of immunohistochemical staining methods for the detection of prostate cancer cells in bone marrow.

Publications:

Takai DK, Craighead, N, Saini, A, **Celniker, A.**, Burkly, L.C., Lee, K.P., Chute, J.P., Harlan, D.M. and Kirk, A.D. Costimulatory molecules are active in the human xenoreactive T-cell response but not in natural killer-mediated cytotoxicity. Transplantation. 2000 Jul 15;70(1):162-7.

Modi, N., Baughman, S., Paasch, B., **Celniker, A.**, and Smith, S. Pharmacokinetics and pharmacodynamics of TP-9201, a GPIIb/IIIa antagonist, in rats and dogs. J Cardiovasc Pharmacol. 1995 Jun; 25(6): 888-97.

Boguniewicz, M., Martin, R., Martin, D., Gibson, U., **Celniker, A.**, Williams, M., and Leung, D. The effects of nebulized recombinant interferon-gamma in asthmatic airways. J Allergy Clin Immunol. 1995 Jan;95(1 Pt 1):133-5

Martin, R., Boguniewicz, M., Hensen, J.E., **Celniker, A.**, Williams, M., Giorno, R., and Leung, D.Y. The Safety and Effects of Inhaled Interferon Gamma in Normal Human Airways. Am Rev of Resp Dis 148: 1677-1682, 1993.

Hartman, M.L., Clayton, P.E., Johnson M.L. Perlman, A.J., **Celniker, A.C.**, Alberti, K.G.M.M. and Thoner, M.O. A Low-Dose Euglycemic Infusion of Recombinant Human Insulin-Like

Growth Factor I Rapidly Suppresses Fasting-Enhanced Pulsatile Growth Hormone Secretion in Humans. JCI 91: 2453-2462, 1993.

Stewart, D.R., Overstreet, J.W., **Celniker, A.C.**, Hess, D.L., Cragun, J.R., Boyers, S.P. and Lasley, B.L. The Relationship Between HCG and Relaxin Secretion in Normal Pregnancies vs Periimplantation Spontaneous Abortions. Clin Endo 38: 379-385, 1993.

Simon, C.J.Y., Underwood, L.E., **Celniker, A.** and Clemmons, D.R. 1992. Effects of Recombinant Insulin-Like Growth Factor-I (IGF-I) and Growth Hormone on Serum IGF-Binding Proteins in Calorically Restricted Adults. J. Clin Endocrinol. Metab. 75: 603-608, 1992

Lieberman, SA, Bukar, J, Chen, SA, **Celniker, AC**, Compton, PG, Cook, J, Albu, J, Perlman, AJ, and Hoffman, AR. 1992. Effects of Recombinant Human Insulin-Like Growth Factor-I (rhIGF-I) on Total and Free IGF-I Concentrations, IGF Binding Proteins, and Glycemic Response in Humans. J. Clin Endocrinol Metab. 75: 30-36, 1992.

Albini, C.H., Sotos, J., Sherman, B., Johanson, A., **Celniker, A.**, Hopwood, N., Quattrin, T., Mills, B., and MacGillivray, M.H. 1991. Diagnostic Significance of Urinary Growth Hormone Measurements in Children with Growth Failure: correlation between serum and urine GH. Pediatric Research 29: 619-622, 1991.

Ferraiolo, B.L., Winslow, J., Laramée, G., **Celniker, A** and Paul Johnston. : The Pharmacokinetics and Metabolism of Human Relaxins in Rhesus Monkeys. Pharmaceutical Research, 8: 1032-1038, 1991.

Girard, J., **Celniker, A.**, Price, A., Tanaka, T., Walker, J., Welling, K., and Albertsson-Wikland, K. Urinary Measurement of Growth Hormone Secretion. Acta Paediatr Scand [Suppl], 366: 149-154, 1990.

Stewart, D.R., **Celniker, A.C.**, Taylor, C.A., Cragun, J.R., Overstreet, J.W. and Lasley, B.L. Relaxin in the Peri-Implantation Period. J. Clin Endocrinol and Met, 70: 1771, 1990

Celniker A.C., Chen, A.B., Wert, R.M. and Sherman, B.M. Variability in the Quantitation of Circulation Growth Hormone Using Commercial Immunoassays. J. Clin Endocrinol and Met, 68: 469, 1989

Ahmann, R.R., Garewal, H.S., Schiffman, R., **Celniker, A.** and Rodney, S. Intracellular Adenosine Triphosphate as a Measure of Human Tumor Cell Viability and Drug Modulated Growth. J. In Vitro Cell Dev Biol 23: 474, 1987

Montgomery, D.W., **Celniker, A.** and Zukoski, C.F. Didemnin B--An Immunosuppressive Cyclic Peptide that Stimulates Murine Hemagglutinating Antibody Responses and Induces Leukocytosis In Vivo. Transplantation 43: 133, 1987

Nagle, R.B., Ahmann, F.R., McDaniel, K.M., Paquin, M.L., Clark, V.A. and **Celniker, A.** Cytokeratin Characterization of Human Prostatic Carcinoma and Its Derived Cell Lines. Cancer Res 47: 281, 1987

Garewal, H.S., Ahmann, F.R., Schiffman, R.B. and **Celniker, A.** ATP Assay: Ability to Distinguish Cytostatic from Cytocidal Anticancer Drug Effects. JNCI 77: 1039, 1986

Abstracts:

Elias, K, Wilson, M, Fehskens, E, Giltinan, D, **Celniker, A**, Harrison, D, Tanner, J and Cronin, M. Recombinant Human Growth Hormone Sustains Elevated Insulin-Like Growth Factor Levels in Immature Rhesus Monkeys. 74th Annual Meeting of the Endocrine Society, San Antonio Texas, June 24-27, 1992, pg. 225.

Harrison, D, **Celniker, A**, Reifsnyder, D, Sipes, D, Schroeder, K and Gesundheit, N. Measurement of Serum Insulin-Like Growth Factor Binding Protein 3 Using a Two Site ELISA. 74th Annual Meeting of the Endocrine Society, San Antonio Texas, June 24-27, 1992, pg. 295.

Celniker, AC and Chen, AB. The Use of Antigen ELISAS in the Development of Protein and Peptide Pharmaceuticals. 25 Years of Immunoenzymatic Techniques, International Congress, Athens Greece, September 9-12, 1991.

Albini, CH, **Celniker, A**, Quattrin, T, Fehskens, E, and MacGillivray, M. Comparison of Enzyme Linked Immunosorbent Assay (ELISA) Versus Radioimmunoassay (RIA) Methods for Quantitation of Urinary Growth Hormone (GH) Output. 73rd Annual Meeting of the Endocrine Society, Washington, DC, June 19-11, 1991, pg. 90.

Wong, J., **Celniker, A.**, Le Thi, P. and Carlton, E. Development of Age and Sex Related Reference Values for IGF-I and Comparison to Levels During Treatment with Recombinant hGH for GHD. 2nd International Symposium on Insulin-Like Growth Factors/Somatomedins, San Francisco, California, January 12-16, 1991, pg. 96.

Anicetti, V., Smith, C., Pocekay, J. and **Celniker, A.** Automated Assay Methods for the Measurement of Free and Total IGF-I in Human Plasma. 2nd International Symposium on Insulin-Like Growth Factors/Somatomedins, San Francisco, California, January 12-16, 1991, pg. 99.

Lieberman, S.A., Chen, S.A., Albu, J., Legaspi, B.V., Maneval, D.C., **Celniker, A.C.**, Compton, P., Bukar, J., Hoffman, A.R., and Perlman, A.J. Effects of Multiple Injections of rhIGF-I on Free IGF-I Disposition and Glycemic Response in Humans. 2nd International Symposium on Insulin-Like Growth Factors/Somatomedins, San Francisco, California, January 12-16, 1991, pg. 271.

Celniker, A.C., Chen, S., Spanski, N., Pocekay, J., and Perlman, A.J. IGF-I: Assay Methods and Pharmacokinetics of Free IGF-I in the Plasma of Normal Human Subjects Following IV Administration. 72nd Annual Meeting of the Endocrine Society, Atlanta, Georgia, June 20-23, 1990, pg. 310.

Stewart, D.R., **Celniker, A.C.** Taylor, C.A., Cragun, J.R. Overstreet, J.W. and Lasley, B.L. Relaxin in Transient Early Human Pregnancies. 72nd Annual Meeting of the Endocrine Society, Atlanta, Georgia, June 20-23, 1990, pg. 204

Fehskens, E.F., Fletcher, B.L. and **Celniker, A.C.** A Sensitive ELISA for the Quantitation of hGH in Urine. 71st Annual Meeting of the Endocrine Society, Seattle, Washington, June 21-24, 1989, pg. 445

Bregman, M.D., **Celniker, A.**, Funk, C. and Meyskens, F.L., Biological Enhancers of Tumor Necrosis Factor Cytotoxicity of Human Melanoma by Cathespsin D. Seventy-seventh Annual Meeting of the American Association for Cancer Research, Los Angeles, California, May 7-10, 1986, Proc Am Assoc Cancer Res Annual Meet. 27: 379.

Garewal, H.S., Ahmann, F.R. and **Celniker, A.** The ATP Assay Ability to Distinguish Cytostatic from Cytocidal Anti-Cancer Drug Effects. Eighty-seventh Annual Meeting of the American Society for Clinical Pharmacology and Therapeutics, Washington, D.C., March 20-22, 1986, Clin Pharmacol Ther, 39: 194.

Celniker A. and Lucas, D. Identification of Different forms of Human Cathepsin D with Monoclonal Antibodies. 70th Annual Meeting of the Federation of American Societies for Experimental biology, St. Louis, MO., April 13-18, 1986. Fed Proc 45: 362

Celniker, A. and Lucas, D.O. Cathepsin D in Differentiating U-937 Cells. 69th Annual Meeting of the Federation of American Societies for Experimental Biology, Anaheim, CA., April 21-26, 1985. Fed Proc, 44: 1702

19th
EDITION

**Remington: The
Science and Practice
of Pharmacy**

Volume II

72

1
2
3
4
5
6
7
8

9
10
11
12
13
14
15
16
17
18
19
20
21
22

23
24
25
26
27
28

P
29
30
31
32
33
34
35
36
37
38
39

40
41
42
43
44
45
46
47
48
49
50

Entered according to Act of Congress, in the year 1885 by Joseph P Remington,
in the Office of the Librarian of Congress, at Washington DC

Copyright 1889, 1894, 1905, 1907, 1917, by Joseph P Remington

Copyright 1926, 1936, by the Joseph P Remington Estate

Copyright 1948, 1951, by The Philadelphia College of Pharmacy and Science

Copyright 1956, 1960, 1965, 1970, 1975, 1980, 1985, 1990, 1995, by The Philadelphia College of
Pharmacy and Science

All Rights Reserved

Library of Congress Catalog Card No. 60-53334

ISBN 0-912734-04-3

*The use of structural formulas from USAN and the USP Dictionary of Drug Names is by
permission of The USP Convention. The Convention is not responsible for any inaccuracy
contained herein.*

*NOTICE—This text is not intended to represent, nor shall it be interpreted to be, the equivalent
of or a substitute for the official United States Pharmacopeia (USP) and / or the National
Formulary (NF). In the event of any difference or discrepancy between the current official
USP or NF standards of strength, quality, purity, packaging and labeling for drugs and
representations of them herein, the context and effect of the official compendia shall prevail.*

Printed in the United States of America by the Mack Printing Company, Easton, Pennsylvania

CHAPTER 86

Solutions, Emulsions, Suspensions and Extracts

J G Nairn, PhD

Professor of Pharmacy
Faculty of Pharmacy
University of Toronto
Toronto, Canada M5S 1A1

The dosage forms described in this chapter may be prepared by dissolving the active ingredient(s) in an aqueous or nonaqueous solvent, by suspending the drug (if it is insoluble in pharmaceutically or therapeutically acceptable solvents) in an appropriate medium or by incorporating the medicinal agent into one of the two phases of an oil and water system. Such solutions, suspensions and emulsions are further defined in subsequent paragraphs but some, with similar properties, are considered elsewhere. These dosage forms are useful for a number of reasons. They can be formulated for different routes of administration: oral use, introduction into body cavities or applied externally. The dose easily can be adjusted by dilution, and the oral liquid form readily can be administered to children or people unable to swallow tablets or capsules. Extracts eliminate the need to isolate the drug in pure form, allow several ingredients to be administered from a single source (eg, pancreatic extract) and permit the preliminary study of drugs from natural sources. Occasionally, solutions of drugs such as potassium chloride are used to minimize adverse effects in the gastrointestinal tract.

The preparation of these dosage forms involves several considerations on the part of the pharmacist: purpose of the drug, internal or external use, concentration of the drug, selection of the liquid vehicle, physical and chemical stability of the drug, preservation of the preparation and use of appropriate excipients such as buffers, solubilizers, suspending agents, emulsifying agents, viscosity controlling agents, colors and flavors. Oral preparations require that consideration be given to improving patient compliance by making an acceptable product; consequently, color, odor and taste must be considered. These organoleptic factors are described in Chapter 80. The viscosity of a product also must be considered in order that it has the proper palatability for an oral preparation and to have the appropriate suspending properties if it is an emulsion or suspension. The theory pertaining to these systems is provided in Chapters 21 and 22. The theory of solutions, which involves solubility, ionization, pH control through the use of buffers and solubilization, is discussed in Chapters 16 and 17. Because of the complexity of some manufactured products, compounding may be carried out with the aid of linear programming models in order to obtain the optimal product. Chapters (87 to 89) should be consulted for information on the preparation and characteristics of those liquid preparations that are intended for ophthalmic or parenteral use.

Much has been written during the past decade about the biopharmaceutical properties of, in particular, the solid dosage forms. In assessing the bioavailability of drugs in tablets and capsules, many researchers first have studied the absorption of drugs administered in solution. Since drugs are absorbed in their dissolved state, frequently it is found that the absorption rate of oral dosage forms decreases in the following order: aqueous solution > aqueous suspension > tablet or capsule. The bioavailability of a medicament, for oral ingestion and absorption, should be such that eventually all of the drug is absorbed as it passes through the gastrointestinal tract, regardless of the dosage form. Some formulation fac-

tors which may influence the bioavailability and pharmacokinetics of drugs in solution include concentration of the drug, volume of liquid administered, pH, buffer capacity and viscosity. Emulsions and suspensions are more complex systems and consequently the extent of absorption and pharmacokinetic parameters may be affected by a number of additional formulation factors such as surfactants, type of viscosity agent, particle size and particle-size distribution, polymorphism and solubility of drug in the oil phase. Specific examples are provided in Chapter 19. There are a number of reasons for formulating drugs in forms in which the drug is not in the molecular state. These are improved stability, improved taste, low water solubility, palatability and ease of administration. It becomes apparent, then, that each dosage form will have advantages and disadvantages.

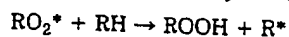
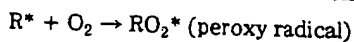
Liquid preparations may be dispensed in one of three ways. The pharmacist may dispense the product in its original container, buy the product in bulk and repackage it at the time a prescription is presented by the patient or compound the solution, suspension or emulsion in the dispensary. Compounding may involve nothing more than mixing marketed products in the manner indicated on the prescription or, in specific instances, may require the incorporation of active ingredients in a logical and pharmaceutically acceptable manner into the aqueous or nonaqueous solvents which will form the bulk of the product.

The pharmacist, in the first instance, depends on the pharmaceutical manufacturer to produce a product that is effective, elegant and stable when stored under reasonably adverse conditions. Most manufacturers attempt to guarantee efficacy by evaluating their products in a scientifically acceptable manner but, in some instances, such efficacy is relative. For example, cough mixtures marketed by two different manufacturers may contain the same active ingredients and it becomes difficult to assess the relative merits of the two products. In such instances the commercial advantage gained by one over the other may be based on product acceptability and preference which includes such factors as color, odor, taste, pourability, uniformity and packaging. Two additional important factors which must be considered in formulations are the stability of active and other ingredients, and the prevention of microbial contamination.

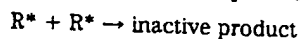
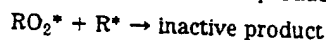
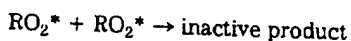
The stability of the active ingredient in the final product is of prime concern to the formulator. In general, drug substances are less stable in aqueous media than in the solid dosage form and it is important, therefore, to properly stabilize and preserve, in particular those solutions, suspensions and emulsions that contain water. Certain simple chemical reactions can occur in these products. These may involve an ingredient-ingredient interaction which implies a poor formulation, a container-product interaction which may alter product pH and thus, for pH-sensitive ingredients, be responsible for the subsequent formation of precipitates or a direct reaction with water, ie, hydrolysis. The stability of pharmaceutical products is discussed in Chapter 38.

The more complicated reactions usually involve oxygen. Vitamins, essential oils and almost all fats and oils can be oxidized. Formulators usually use the word *autoxidation*

when the ingredient(s) in the product react with oxygen but without drastic external interference. Such reactions first must be initiated by heat, light (including ultraviolet radiant energy), peroxides or other labile compounds or heavy metals such as copper or iron. This initiation step results in the formation of a free radical (R^*) which then reacts with oxygen.



The free radical thus is regenerated and reacts with more oxygen. This propagation step is followed by the termination reactions.



The effect of trace metals can be minimized by using citric acid or EDTA i.e., sequestering agents. Antioxidants, however, may retard or delay oxidation by reacting with the free radicals formed in the product. Examples of antioxidants are the propyl, octyl and dodecyl esters of gallic acid, butylated hydroxyanisole (BHA) and the tocopherols or vitamin E. For a more detailed approach to the prevention of oxidative deterioration in pharmaceuticals, the information provided by Connors *et al.*¹ should be consulted. A description of many antioxidants is given in Chapter 80.

The problem of drug stability has been well-defined by pharmaceutical scientists, but during the past few years a secondary and, in some respects, more serious problem has confronted the manufacturer of liquid preparations. Such pharmaceutically diverse products as baby lotions and milk of magnesia have been recalled from the market because of microbial contamination. In a survey of retail packages of liquid antacid preparations containing magnesium hydroxide, it was found that 30.5% of the finished bottles were contaminated with *Pseudomonas aeruginosa*. The aerobic plate count ranged from less than 100 to 9,300,000 organisms/g. Kurup and Wan² describe many preparations that are not preserved adequately and thus are not able to resist microbial contamination. Other examples could be cited but the range of microorganisms which can contaminate the liquid preparation includes the *Salmonella* sp, *E. coli*, certain *Pseudomonas* sp, including *P. aeruginosa*, and *Staphylococcus aureus*. Bruch³ describes the types of microorganisms found in various products and attempts to evaluate the hazards associated with the use of nonsterile pharmaceuticals. Coates⁴ in a series of papers describes various interactions which must be considered when preservatives are selected.

The USP recommends that certain classes of products be tested for microbial count and for specified indicator microbial contaminants, eg, natural plant, animal and some mineral products, for freedom from *Salmonella* sp; oral solutions and suspensions, for freedom from *E. coli*; articles applied topically, for freedom from *P. aeruginosa* and *S. aureus* and articles for rectal, urethral or vaginal administration, for yeasts and molds.

Products may become contaminated for a number of reasons.

The raw materials used in the manufacture of solutions, suspensions and emulsions are excellent growth media for bacteria. Water, in particular, must be handled with care but substances such as gums, dispersing agents, surfactants, sugars and flavors can be the carriers of bacteria which ultimately contaminate the product.

Equipment. Bacteria grow well in the nooks and crevices of pharmaceutical equipment (and in the simple equipment used in the dispensary). Such equipment should be cleaned thoroughly prior to use.

Environment and personnel can contribute to product contamination. Hands and hair are the most important carriers of contaminants. General cleanliness thus is vital. Head coverings must be used by those involved in the manufacturing process and face masks should be used by those individuals suffering from colds, coughs, hay fever and other allergic manifestations.

Packaging should be selected so that it will not contaminate the product and also will protect it from the environment.

Finally, consumer use may result in the introduction of microorganisms as a source of contamination, and this is of particular concern if the organism is pathogenic. The consumer should be instructed in the proper technique in order to minimize contamination, and the manufacturer should ensure, through the use of suitable challenge tests, that the product is preserved appropriately and will reduce a severe microbial challenge.

Most factors cited above relate to good manufacturing practice. However, the formulator should add a preservative to the product and decrease the probability of product contamination. If the product contains water, which is an important requirement for microbial growth, it almost is mandatory to include a preservative in the formulation. Nearly all products described in this chapter contain water and, thus, with certain exceptions, eg, aqueous acids, will support microbial growth. Microbes will grow in an aqueous solution, and in the aqueous phase of multiphase systems such as emulsions and suspensions. It must be stressed that the addition of an appropriate preservative in no way replaces good manufacturing practice but merely provides further assurance that the product will retain its pharmaceutically acceptable characteristics until it is used by the patient and for sometime thereafter.

The major criteria that should be considered in selecting a preservative are as follows: it should be effective against a wide spectrum of microorganisms, stable for its shelf life, nontoxic, nonsensitizing, compatible with the ingredients in the dosage form inexpensive and essentially relatively free of taste and odor.

In addition to the above discussion, there are a number of specific factors which should be taken into account when a preservative is selected:

1. The site of use, eg, external, internal or ophthalmic.
2. The pH of the liquid, as it may affect both the ionization of the preservative and its stability.
3. The solvent, as this will affect the solubility of the preservative.
4. Partitioning into the oil phase of an emulsion, thereby reducing the concentration in the aqueous phase where preservative action takes place.
5. Adsorption onto the solid phase of a suspension, thereby reducing the concentration in the aqueous phase.
6. Processing and packaging variables such as heat, order of addition of the ingredients, stirring or container materials.
7. Type of dosage form, eg, solution, emulsion or suspension.

Preservatives^{5,6} may be grouped into a number of classes depending upon their molecular structure and only a few will be discussed. The reader should consult Chapter 80 or selected texts in the bibliography for further description.

Alcohols—Ethanol is useful as a preservative when it is used as a solvent; however, it does need a relatively high concentration, somewhat greater than 10%, to be effective. Too high a concentration may result in incompatibilities in suspension and emulsion systems. Propylene glycol also is used as a solvent in oral solutions and topical preparations, and it can function as a preservative in the range of 15 to 30%. It is not volatile like ethanol and is used frequently not only in solutions but also in suspensions and emulsions. Other alcohols used in lower concentrations, about 1%, for preservative action, include chlorobutanol and phenylethyl alcohol.

Acids—Benzoic acid has a low solubility in water, about 0.34% at 25°. The concentration range used for inhibitory action varies from 0.1% to 0.4%. Only the nonionized form is effective and therefore its use is restricted to preparations with a pH below 4.5. Sorbic acid also has a low solubility in water, 0.3% at 30°. Suitable concentrations for preservative action are in the range of 0.05 to 2%. Its preservative action is due to the nonionized form; consequently, it is only effective in acid media. Because of the double bond in its structure, it is subject to oxidation.

Esters—Parabens are esters of *p*-hydroxybenzoic acid and include the methyl, ethyl, propyl and butyl derivatives. The solubility in water decreases as the molecular weight increases from 0.25% for the methyl ester to 0.02% for the butyl ester. These compounds are used widely in pharmaceutical products and are effective and stable over a pH range of 4 to 8. They are employed at concentrations up to about 0.2%. Frequently, two esters are used in combination in the same preparation. This achieves a higher total concentration, and the mixture tends to be active against a wider range of microorganisms. Their activity is reduced in the presence of nonionic surface active agents due to binding. In alkaline solutions, ionization takes place and this reduces their activity; in addition, hydrolytic decomposition of the ester group occurs with a loss of activity.

Qu
mixt
prese
depe
com
stabl
prese
surfa
prepa
with
It
pens
sibil
tena
stab
ied i
be r
indic
in th
resis
evid
syru
or c
Emu

A
disse
sents
solut
the s
phys
phys
narrow
water
visc
inclu
ignat
lions
Nasc

Wate

Th
herei
for th
lessn
colog
howe
that i
care.
with
com
musl
For fi
Fin
in the
Puri
oper
assay
Such
exch
A
prod
the s
water
provi
the r
subje

Quaternary Ammonium Compounds—Benzalkonium chloride is a mixture consisting principally of the homologs $C_{12}H_{25}$ and $C_{14}H_{29}$. This preservative is used at a relatively low concentration, 0.002 to 0.02%, depending on the nature of the pharmaceutical product. This class of compounds has an optimal activity over the pH range of 4 to 10 and is quite stable at room temperature. Because of the cationic nature of this type of preservative, it is incompatible with many anionic compounds such as surfactants and can bind to nonionic surfactants. It is used generally in preparations for external use or those solutions which come in contact with mucous membranes.

It now should be obvious that when the pharmacist dispenses or compounds the various liquid preparations responsibility is assumed along with the manufacturer, for the maintenance of product stability. The USP includes a section on stability considerations in dispensing, which should be studied in detail. Certain points are self-evident. Stock should be rotated and replaced if expiration dates on the label so indicate. Products should be stored in the manner indicated in the compendium; eg, in a cool place or a tight, light-resistant container. Further, products should be checked for evidence of instability. With respect to solutions, elixirs and syrups, color change, precipitation and evidence of microbial or chemical gas formation are major signs of instability. Emulsions may cream but if they break (ie, there is a separa-

tion of an oil phase) the product is considered to be unstable. Sedimentation and caking are primary indications of instability in suspensions. The presence of large particles may mean that excessive crystal growth has occurred.

The USP states that if the product must be repackaged, care and the container specified by the compendium must be used. For example, a suitably opaque plastic container should be used if a light-resistant container is specified. If a product is diluted, or where two products are mixed, the pharmacist should use his or her knowledge to guard against incompatibility and instability. Oral antibiotic preparations constituted into liquid form should never be mixed with other products. If the chemical stability of extemporaneously prepared liquid preparations is unknown, their use should be minimized and every care taken to insure that product characteristics will not change during the time it must be used by the patient.

Because of the number of excipients and additives in these preparations, it is recommended that all the ingredients be listed on the container to reduce the risks which confront hypersensitive patients when these products are administered. Finally, the pharmacist should inform the patient regarding the appropriate use of the product, the proper storage conditions and the time after which it should be discarded.

Solutions

Aqueous Solutions

A solution is a homogeneous mixture that is prepared by dissolving a solid, liquid or gas in another liquid and represents a group of preparations in which the molecules of the solute or dissolved substance are dispersed among those of the solvent. Solutions also may be classified on the basis of physical or chemical properties, method of preparation, use, physical state, number of ingredients and particle size. The narrower definition in this subsection limits the solvent to water and excludes those preparations that are sweet and/or viscous in character and nonaqueous solutions. This section includes, therefore, those pharmaceutical forms that are designated as *Water*, *Aromatic Waters*, *Aqueous Acids*, *Solutions*, *Douches*, *Enemas*, *Gargles*, *Mouthwashes*, *Juices*, *Nasal Solutions*, *Otic Solutions* and *Irrigation Solutions*.

Water

The major ingredient in most of the dosage forms described herein is water. It is used both as a vehicle and as a solvent for the desired flavoring or medicinal ingredients. Its tastelessness, freedom from irritating qualities and lack of pharmacological activity make it ideal for such purposes. There is, however, a tendency to assume that its purity is constant and that it can be stored, handled and used with a minimum of care. While it is true that municipal supplies must comply with Environmental Protection Agency (EPA) regulations (or comparable regulations in other countries), drinking water must be repurified before it can be used in pharmaceuticals. For further information on water, see Chapter 23.

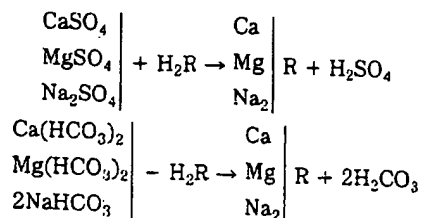
Five of the six solvent waters described in the USP are used in the preparation of parenterals, irrigations or inhalations. *Purified Water* must be used for all other pharmaceutical operations, dosage forms and, as needed, in all USP tests and assays. It must meet rigid specifications for chemical purity. Such water may be prepared by distillation, by use of ion-exchange resins or by reverse osmosis.

A wide variety of commercially available stills are used to produce distilled water. The end use of the product dictates the size of the still and extent of pretreatment of the drinking water introduced into the system. A description of stills is provided in Chapter 87. Such water may be sterile provided the condenser is sterile, but to be called sterile it must be subjected to a satisfactory sterilization process. However, it

has been shown that *P. aeruginosa* (and other microorganisms) can grow in the distilled water produced in hospitals. The implications of this are obvious. Sterile water may be sterile at the time of production but may lose this characteristic if it is stored improperly. Hickman *et al.*,⁷ by regrouping the components of conventional distillation equipment, have described a method for the continuous supply of sterile, ultrapure water. Quality-control procedures for monitoring the microbiological quality of water should be performed in the pharmaceutical manufacturer's production facilities.

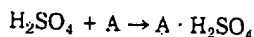
The major impurities in water are calcium, iron, magnesium, manganese, silica and sodium. The cations usually are combined with the bicarbonate, sulfate or chloride anions. "Hard" waters are those that contain calcium and magnesium cations. Bicarbonates are the major impurity in "alkaline" waters.

Ion-exchange (deionization, demineralization) processes will remove most of the major impurities in water efficiently and economically. A cation exchanger, H_2R , first converts bicarbonates, sulfates and chlorides to their respective acids, eg,

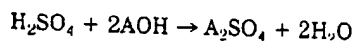


Carbonic acid decomposes to carbon dioxide (which is removed by aeration in the decarbonator) and water.

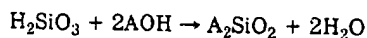
The anion exchanger may contain either a weakly basic or a strongly basic anion resin. These adsorb sulfuric, hydrochloric and nitric acids. Chemical reactions may involve complete adsorption or an exchange with some other anion.



If the resin contains a hydroxyl group, water is formed during the purification process.



Weakly dissociated carbonic and silicic acids can be removed only by strongly basic anion resins.



Unit capacity varies with the nature of the installation, but it is possible to process as much as 15,000 gal of water/min.

Deionization processes do not necessarily produce *Purified Water* which will comply with EPA requirements for drinking water. Resin columns retain phosphates and organic debris. Either alone or in combination, these substances can act as growth media for microorganisms. Observations have shown that deionized water containing 90 organisms/mL contained, after 24-hour storage, 10^6 organisms/mL. Columns can be cleaned partially of pseudomonads by recharging, but a 0.25% solution of formaldehyde will destroy most bacteria. The column must be washed thoroughly and checked for the absence of aldehyde (with a Schiff's Reagent) before it can be used to generate deionized water.

Ultraviolet radiant energy (240–280 nm), heat or filtration can be used to limit the growth, kill or remove microorganisms in water. The latter method employs membrane filters and can be used to remove bacteria from heat-labile materials as described under membrane filters in Chapter 84.

The phenomenon of osmosis involves the passage of water from a dilute solution across a semipermeable membrane to a more concentrated solution. Flow of water can be stopped by applying pressure, equal to the osmotic pressure, to the concentrated solution. The flow of water can be reversed by applying a pressure, greater than the osmotic pressure. The process of reverse osmosis utilizes the latter principle; by applying pressure, greater than the osmotic pressure, to the concentrated solution, eg, tap water, pure water may be obtained (see *Reverse Osmosis* in Chapter 37).

Cellulose acetate is used in the manufacture of semipermeable membranes for purifying water by reverse osmosis. This polymer has functional groups that can hydrogen-bond to water or other substances such as alcohol. The water molecules which enter the polymer are transported from one bonding site to the next under pressure. Because of the thin layer of pure water strongly adsorbed at the surface of the membrane, salts, to a large extent, are repelled from the surface, the higher-valent ions being repelled to a greater extent, thus causing a separation of ions from the water. Organic molecules are rejected on the basis of a sieve mechanism related to their size and shape. Small organic molecules, with a molecular weight smaller than approximately 200, will pass through the membrane material. Since there are few organic molecules with a molecular weight of less than 200 in the municipal water supply, reverse osmosis usually is sufficient for the removal of organic material. The pore sizes of the selectively permeable reverse-osmosis membranes are between 5 and 100 Å. Viruses and bacteria larger than 100 Å are rejected if no imperfections exist in the membrane. The membranes may and do develop openings which permit the passage of microorganisms. Because of the semistatic conditions, bacteria can grow both upstream and downstream of the membrane. Improvements in membranes are being made continually in type and manufacturing process such as the use of polyamide materials. It is expected that the preparation of water with negligible or no bacteria present will be achieved by this process.

The selection of water-treatment equipment depends upon the quality of water to be tested, the quality of water required and the specific pharmaceutical purpose of the water. Frequently, two or more methods are used to produce the water desired, for example, filtration and distillation, or filtration, reverse osmosis and ion exchange.

Aromatic Waters

Aromatic waters, known also as medicated waters, are clear, saturated aqueous solutions of volatile oils or other aromatic or volatile substances. Their odors and tastes are similar to those of the drugs or volatile substances from which they are

prepared. They are used principally as flavored or perfumed vehicles. Aromatic Waters may be prepared by distillation or solution of the aromatic substance with or without the use of a dispersing agent such as talc. Peppermint Water USP and Stronger Rose Water USP are examples of aromatic waters.

Other methods have been suggested for preparing aromatic waters based on the use of soluble concentrates or on incorporation of solubilizing agents such as polysorbate 20.

Concentrated waters eg, peppermint, dill, cinnamon and caraway, may be prepared as follows:

Dissolve 20 mL of the volatile oil in 600 mL of 90% ethanol. Add sufficient purified water in successive small portions to produce 1000 mL. Shake vigorously after each addition. Add 50 g of sterilized purified talc, shake occasionally for several hours and filter.

The aromatic water is prepared by diluting the concentrate with 39 times its volume of water.

The chemical composition of many of the volatile oils is known and suitable synthetic substances may be used in preparing pharmaceuticals and cosmetics. Similarly, many synthetic aromatic substances have a characteristic odor; eg, geranyl phenyl acetate has a honey odor. Such substances, either alone or in combination, can be used in nonofficial preparations. Additional information regarding the appropriate preparation of aromatic waters is provided in RPS-18, Chapter 83, and RPS-17, Chapter 84.

The principal difficulty experienced in compounding prescriptions containing aromatic waters is due to a "salting out" action of certain ingredients, such as very soluble salts, on the volatile principle of the aromatic water. A replacement of part of the aromatic water with purified water is permissible when no other function is being served than that of a vehicle.

Preservation—Aromatic waters will deteriorate with time and should, therefore, be made in small quantities and protected from intense light, excessive heat and stored in airtight, light-resistant containers.

Aqueous Acids

The official inorganic acids and certain organic acids, although of minor significance as therapeutic agents, are of great importance in chemical and pharmaceutical manufacturing. This is especially true of acetic, hydrochloric and nitric acids.

Percentage Strengths—Many of the more important inorganic acids are available commercially in the form of concentrated aqueous solutions. The percentage strength varies from one acid to another and depends on the solubility and stability of the solute in water and on the manufacturing process. Thus, the official Hydrochloric Acid contains from 36.5 to 38% by weight of HCl, whereas Nitric Acid contains from 69 to 71% by weight of HNO_3 .

Because the strengths of these concentrated acids are stated in terms of % by weight, it is essential that specific gravities also be provided if one is to be able to calculate conveniently the amount of absolute acid contained in a unit volume of the solution as purchased. The mathematical relationship involved is given by the equation $M = V \times S \times F$, where M is the mass in g of absolute acid contained in V mL of solution having a specific gravity S and a fractional percentage strength F . As an example, Hydrochloric Acid containing 36.93% by weight of HCl has a specific gravity of 1.1875. Therefore, the amount of absolute HCl supplied by 100 mL of this solution is given by:

$$M = 100 \times 1.1875 \times 0.3693 = 43.85 \text{ g HCl}$$

Incompatibilities—Although many of the reactions characteristic of acids offer opportunities for incompatibilities, only a few are of sufficient importance to require more than casual mention. Acids and acid salts decompose carbonates with liberation of carbon dioxide and, in a closed container, sufficient pressure may be developed to produce an explosion. Inorganic acids react with salts of organic acids to produce the free organic acid and a salt of the inorganic acid. If in-

solu
acid
lates
conc
cert
insol
vert
solu
Th
orga
in pr
It
elixi
free:
patil
Ac
they
frequ
the s
Di
solu
Dilu
for th
Th
as p
dilu
becc
conc
mak
tion
dilu

Thur
Acid
(sp g

Di
men
mer
The
men
than

Solu

A
that
solu
tion:
eith
is or
such
teris
So
tion
luti
the t
P:
the
into

Sh
the s
suffi
solu
activ
ter).
USP
Ch
than

soluble, the organic acid will be precipitated. Thus, salicylic acid and benzoic acid are precipitated from solutions of salicylates and benzoates. Boric acid likewise is precipitated from concentrated solutions of borates. By a similar reaction, certain soluble organic compounds are converted into an insoluble form. Phenobarbital sodium, for example, is converted into phenobarbital which will precipitate in aqueous solution.

The ability of acids to combine with alkaloids and other organic compounds containing a basic nitrogen atom is used in preparing soluble salts of these substances.

It should be borne in mind that certain solutions, syrups, elixirs and other pharmaceutical preparations, may contain free acid, which causes these preparations to exhibit the incompatibilities characteristic of the acid.

Acids also possess the incompatibilities of the anions which they contain and, in the case of organic acids, these are frequently of prime importance. These are discussed under the specific anions.

Diluted Acids—The diluted acids in the USP are aqueous solutions of acids, of a suitable strength (usually 10% w/v but Diluted Acetic Acid is 6% w/v) for internal administration or for the manufacture of other preparations.

The strengths of the official undiluted acids are expressed as percentages w/w, whereas the strengths of the official diluted acids are expressed as percent w/v. It, therefore, becomes necessary to consider the specific gravities of the concentrated acids when calculating the volume required to make a given quantity of diluted acid. The following equation will give the number of mL required to make 1000 mL of diluted acid:

$$\frac{\text{Strength of diluted acid} \times 1000}{\text{Strength of undiluted acid} \times \text{sp gr of undiluted acid}}$$

Thus, if one wishes to make 1000 mL of Diluted Hydrochloric Acid USP using Hydrochloric Acid which assays 37.5% HCl (sp gr 1.18), the amount required is

$$\frac{10 \times 1000}{37.5 \times 1.18} = 226 \text{ mL}$$

Diluted Hydrochloric Acid USP has been used in the treatment of achlorhydria. However, it may irritate the mucous membrane of the mouth and attack the enamel of the teeth. The usual dose is 5 mL, well-diluted with water. In the treatment of achlorhydria no attempt is made to administer more than a relief-producing dose.

Solutions

A solution, in the present context, is a liquid preparation that contains one or more soluble chemical substances dissolved in water. The solute usually is nonvolatile. Solutions are used for the specific therapeutic effect of the solute, either internally or externally. Although the emphasis here is on the aqueous solution, certain preparations of this type such as syrups, infusions and decoctions have distinctive characteristics and, therefore, are described later in the chapter.

Solvents, solubility and general methods for the incorporation of a solute in a solvent are discussed in Chapter 16. Solutions are usually bottled automatically with equipment of the type shown in Fig. 1.

Preparation—A specific method of preparation is given in the compendia for most solutions. These procedures fall into three main categories.

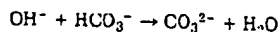
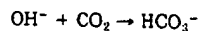
Simple Solutions—Solutions of this type are prepared by dissolving the solute in most of the solvent, mixing until dissolved, then adding sufficient solvent to bring the solution up to the proper volume. The solvent may contain other ingredients which stabilize or solubilize the active ingredient. Calcium Hydroxide Topical Solution USP (Lime Water), Sodium Phosphates Oral Solution USP and Strong Iodine Solution USP are examples.

Calcium Hydroxide Topical Solution contains, in each 100 mL, not less than 140 mg of $\text{Ca}(\text{OH})_2$. The solution is prepared by agitating vigor-

ously 3 g of calcium hydroxide with 1000 mL of cool, purified water. Excess calcium hydroxide is allowed to settle out and the clear, supernatant liquid dispensed.

An increase in solvent temperature usually implies an increase in solute solubility. This rule does not apply, however, to the solubility of calcium hydroxide in water, which decreases with increasing temperature. The official solution is prepared at 25°.

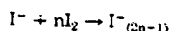
Solutions containing hydroxides react with the carbon dioxide in the atmosphere.



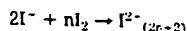
Calcium Hydroxide Topical Solution, therefore, should be preserved in well-filled, tight containers, at a temperature not exceeding 25°.

Strong Iodine Solution contains, in each 100 mL, 4.5–5.5 g of iodine, and 9.5–10.5 g of potassium iodide. It is prepared by dissolving 50 g of iodine in 100 mL of purified water containing 100 g of potassium iodide. Sufficient purified water then is added to make 1000 mL of solution.

One g of iodine dissolves in 2950 mL of water. However, solutions of iodides dissolve large quantities of iodine. Strong Iodine Solution is, therefore, a solution of polyiodides in excess iodide.



Doubly charged anions may be found also



Strong Iodine Solution is used in the treatment of iodide deficiency disorders such as endemic goiter.

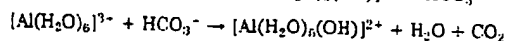
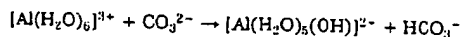
Several antibiotics (eg, cloxacillin sodium, nafcillin sodium and vancomycin), because they are relatively unstable in aqueous solution, are prepared by manufacturers as dry powders or granules in combination with suitable buffers, colors, diluents, dispersants, flavors and/or preservatives. These preparations, Cloxacillin Sodium for Oral Solution, Nafcillin for Oral Solution and Vancomycin Hydrochloride for Oral Solution meet the requirements of the USP. Upon dispensing to the patient, the pharmacist adds the appropriate amount of water. The products are stable for up to 14 days when refrigerated. This period usually provides sufficient time for the patient to complete the administration of all the medication.

Solution by Chemical Reaction—These solutions are prepared by reacting two or more solutes with each other in a suitable solvent. An example is Aluminum Subacetate Topical Solution USP.

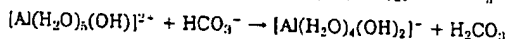
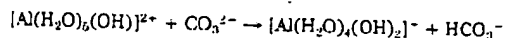
Aluminum sulfate (145 g) is dissolved in 600 mL of cold water. The solution is filtered, and precipitated calcium carbonate (70 g) is added, in several portions, with constant stirring. Acetic acid (160 mL) is added slowly and the mixture set aside for 24 hr. The product is filtered and the magma on the Büchner filter washed with cold water until the total filtrate measures 1000 mL.

The solution contains pentaquoaluminum- and tetraquodihydroxoaluminum (III) acetates and sulfates dissolved in an aqueous medium saturated with calcium sulfate. The solution contains a small amount of acetic acid. It is stabilized by the addition of not more than 0.9% boric acid.

The reactions involved in the preparation of the solution are given below. The hexaquo aluminum cations first are converted to the nonirritating $[\text{Al}(\text{H}_2\text{O})_5(\text{OH})]^{2+}$ and $[\text{Al}(\text{H}_2\text{O})_4(\text{OH})_2]^{+}$ cations.



As the concentration of the hexaquo cations decreases, secondary reactions involving carbonate and bicarbonate occur.



The pH of the solution now favors the precipitation of dissolved calcium ions as the insoluble sulfate. Acetic acid now is added. The bicarbonate which is formed in the final stages of the procedure is removed as carbon dioxide.

Aluminum Subacetate Topical Solution is used in the preparation of Aluminum Acetate Topical Solution USP (Burrow's Solution). The latter solution contains 15 mL of glacial acetic acid, 545 mL of Aluminum Subacetate Topical Solution and sufficient water to make 1000 mL. It is defined as a solution of aluminum acetate in approximately 5%, by weight, of acetic acid in water. It is stabilized by the addition of not more than 0.6% boric acid.

Solution by Extraction—Drugs or pharmaceutical necessities of vegetable or animal origin often are extracted with water or with water containing other substances. Preparations of this type may be classified

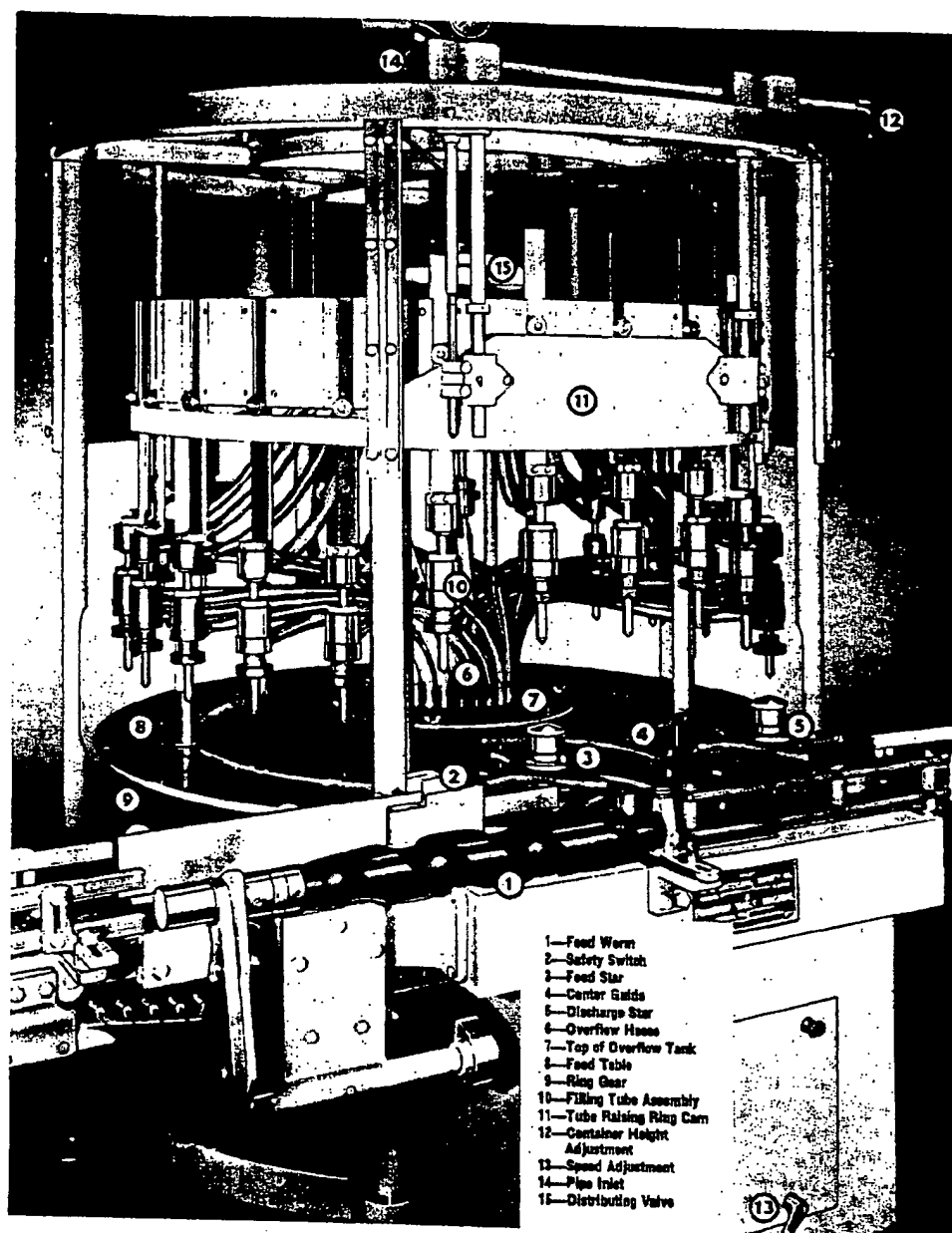


Fig 1. A rotary gravity bottle filler (courtesy, US Bottlers).

as solutions but, more often, are classified as extracts and are described at the end of this chapter.

Douches

A douche is an aqueous solution directed against a part or into a cavity of the body. It functions as a cleansing or antiseptic agent. An *eye douche*, used to remove foreign particles and discharges from the eyes, is directed gently at an oblique angle and allowed to run from the inner to the outer corner of the eye. *Pharyngeal douches* are used to prepare the interior of the throat for an operation and cleanse it in suppurative conditions. Similarly, there are *nasal douches* and *vaginal douches*. Douches usually are directed to the appropriate body part by using bulb syringes (Chapter 107).

Douches most frequently are dispensed in the form of a powder with directions for dissolving in a specified quantity of water (usually warm). However, tablets for preparing solutions are available (eg, Dobell's Solution Tablets) or the solution may be prepared by the pharmacist. If powders or tablets

are supplied, they must be free from insoluble material, in order to produce a clear solution. Tablets are produced by the usual processes (see Chapter 92) but any lubricants or diluents used must be readily soluble in water. Boric acid may be used as a lubricant and sodium chloride normally is used as a diluent. Tablets deteriorate on exposure to moist air and should be stored in airtight containers.

Douches are not official as a class of preparations but several substances in the compendia frequently are employed as such in weak solutions, eg, benzalkonium chloride is used in various douches and Compound Sodium Borate Solution NFXI (Dobell's Solution) has been used as a nasal or pharyngeal douche. A sodium bicarbonate vaginal douche has been used to improve the postcoital test.

Vaginal douches are the most common type of douche and are used for cleansing the vagina and hygienic purposes. Liquid concentrates or powders, which may be prepared in bulk or as single-use packages, should be diluted or dissolved in the appropriate amount of warm water prior to use. The

ingr
age
thyr
Astr
acti
alter
used

Enc

TI
ate
tem
latte
anth
they
grap
So
gen
ener
used
with
prep
water
ema
drat
in e
body
asy

Am
am
inter
mL
such
thes
cro
etha
irrit
othe
daz
of a
drug

St
tre
ing
the
NaH
bate
ben,
mak
and
tor
bani
star
50 g
100
St
othe
ing
Suff
The

Gar

G
tics
ynx
the
is e
prio
sepi
eith
A
has

ingredients used in vaginal douches include antimicrobial agents such as benzalkonium chloride, the parabens or chlorothymol, anesthetics or antipruritics such as phenol or menthol. Astringents such as zinc sulfate or potassium alum, surface-active agents such as sodium lauryl sulfate and chemicals to alter the pH such as sodium bicarbonate or citric acid also are used.

Enemas

These preparations are rectal injections employed to evacuate the bowel (evacuation enemas), influence the general system by absorption or to affect locally the seat of disease. The latter two are called retention enemas. They may possess anthelmintic, nutritive, sedative or stimulating properties, or they may contain radiopaque substances for roentgenographic examination of the lower bowel.

Sodium chloride, sodium bicarbonate, sodium monohydrogen phosphate and sodium dihydrogen phosphate are used in enemas to evacuate the bowel. These substances may be used alone, in combination with each other or in combination with irritants such as soap. Enema of Soap BPC 1963 is prepared by dissolving 50 g of soft soap in sufficient purified water to make 1000 mL of enema. Sodium Phosphate Enema USP contains 6 g of dibasic sodium phosphate heptahydrate and 16 g of monobasic sodium phosphate monohydrate in each 100 mL. Evacuation enemas usually are given at body temperature in quantities of 1 to 2 pt injected slowly with a syringe.

An official retention enema used for systemic purposes is aminophylline. Retention enemas are to be retained in the intestine and should not be used in larger quantities than 150 mL for an adult. Usually, the volume is considerably smaller, such as a few mL. *Microenema* is a term used to describe these small-volume preparations. Vehicles for retention microenemas have been formulated with small quantities of ethanol and propylene glycol, and no significant difference in irritation, as compared with water, was found. A number of other drugs such as valproic acid, indomethacin and metronidazole have been formulated as microenemas for the purpose of absorption. The absorption of large molecular weight drugs, such as insulin, is under current investigation.

Sulfasalazine rectal enema has been administered for the treatment of ulcerative colitis and may be prepared by dispersing the tablets (1-g strength) in 250 mL water. An enema in the form of a suspension is 5-aminosalicylic acid, 168 g; NaH_2PO_4 , 1.6 g; Na_2HPO_4 , 17.9 g; NaCl, 36 g; sodium ascorbate, 2 g; tragacanth, 16 g; methylparaben, 8 g; propylparaben, 2 g; propylene glycol, 100 mL; and distilled water to make 4000 mL. It has been prepared by Montgomery *et al*¹⁸ and shown to be stable for 90 days at both room and refrigerator temperatures. Barium sulfate enema contains 120 g of barium sulfate, 100 mL of acacia mucilage and sufficient starch enema to make 500 mL. An enema containing 30 to 50 g of sodium polystyrene sulfonate has been prepared using 100 mL of sorbitol solution.

Starch enema may be used either by itself or as a vehicle for other forms of medication. A thin paste is made by triturating 30 g of powdered starch with 200 mL of cold water. Sufficient boiling water is added to make 1000 mL of enema. The preparation then is reheated to obtain a transparent liquid.

Gargles

Gargles are aqueous solutions frequently containing antiseptics, antibiotics and/or anesthetics used for treating the pharynx and nasopharynx by forcing air from the lungs through the gargle which is held in the throat; subsequently, the gargle is expectorated. Many gargles must be diluted with water prior to use. Although mouthwashes are considered as a separate class of pharmaceuticals, many are used as gargles, either as is, or diluted with water.

A gargle/mouthwash containing the antibiotic tyrothricin has been shown to provide levels of gramicidin, a component

of tyrothricin, in saliva when used as a gargle rather than a mouthwash. Higher saliva levels of gramicidin were obtained when a lozenge formulation was employed. Rapid relief of pharyngeal and oral pain was obtained when Cepacaine solution, which contains a topical anesthetic, was used as a gargle.

Potassium Chlorate and Phenol Gargle is official in the PC. It contains potassium chlorate, 30 g, patent blue V (Color Index No 42051) commercial food grade (0.01 g), liquified phenol (15 mL) and water for preparations qs to 1000 mL. It should be diluted with 10 volumes of warm water before use. The product should be labeled so that it cannot be mistaken for preparations intended for internal administration.

A flavored solution containing 7.5% povidone-iodine and 35% alcohol (*Isodine*) is available commercially as a mouthwash or gargle after suitable dilution.

Mouthwashes

A mouthwash can be used for two purposes, therapeutic and cosmetic. Therapeutic rinses or washes can be formulated to reduce plaque, gingivitis, dental caries and stomatitis. Cosmetic mouthwashes may be formulated to reduce bad breath through the use of antimicrobial and/or flavoring agents.

Recent information indicates that mouthwashes are being used as a dosage form for a number of specific problems in the oral cavity; for example, mouthwashes containing a combination of antihistamines, hydrocortisone, nystatin and tetracycline have been prepared from commercially available suspensions, powders, syrups or solutions for the treatment of stomatitis, a painful side effect of cancer therapy. Other drugs include allopurinol, also used for the treatment of stomatitis, pilocarpine for xerostoma (dry mouth), tranexamic acid for the prevention of bleeding after oral surgery, amphotericin B for oral candidiasis, chlorhexidine gluconate for plaque control and hexetidine as an antibactericidal and antifungal agent.

Mouthwashes may be used for a number of other purposes; for example, cetylpyridinium chloride and dibucaine hydrochloride mouthwashes provide satisfactory relief of pain in patients with ulcerative lesions of the mouth, mouthwashes or creams containing carbenoxolone are highly effective dosage forms for the treatment of orofacial herpes simplex infections and undetected oral cancer has been recognized using toluidine blue in the form of a mouth rinse.

Mouthwashes generally contain four groups of excipients as suggested by Tricca.⁹

Alcohols—Alcohol is often present in the range of 10–20%. It enhances the flavor, provides a certain sharpness to the taste, aids in masking the unpleasant taste of active ingredients, functions as a solubilizing agent for some flavoring agents and may function as a preservative. Humectants such as glycerin and sorbitol, may form 5–20% of the mouthwash. These agents increase the viscosity of the preparation and provide a certain *body* or *mouth feel* to the product. They enhance the sweetness of the product and, along with the ethanol, improve the preservative qualities of the product.

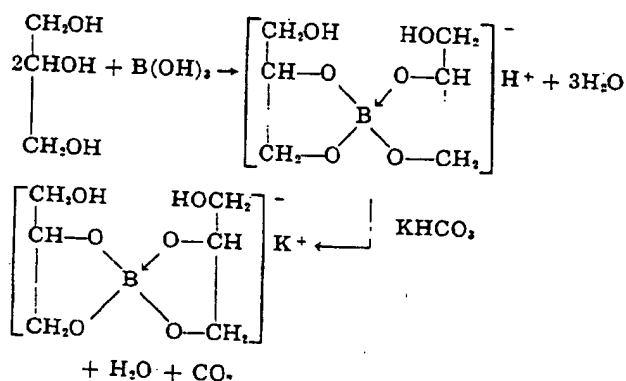
Surfactants, usually of the nonionic class such as polyoxyethylene/polyoxypropylene block copolymers or polyoxyethylene derivatives of sorbitol fatty acid esters may be used. The concentration range is 0.1–0.5%. An anionic surfactant occasionally used is sodium lauryl sulfate. Surfactants are used because they aid in the solubilization of flavors and in the removal of debris by providing foaming action. Cationic surfactants such as cetylpyridinium chloride are used for their antimicrobial properties, but these tend to impart a bitter taste.

Flavors are used in conjunction with alcohol and humectants to overcome disagreeable tastes, and at the same time they must be safe to use. The principle flavoring agents are peppermint, spearmint, cinnamon, wintergreen oils, menthol

or methyl salicylate. Other flavoring agents may be used singly or in combination.

Coloring agents also are used in these products.

The products of commerce (eg, Cepacol, Listerine, Micrin or Scope) vary widely in composition. Antiseptic Solution and Mouthwash are described in NF XII. The latter wash contains sodium borate, glycerin and potassium bicarbonate. The reactions which take place when these substances are dissolved in water are given below.



Compound Sodium Chloride Mouthwash and Zinc Sulphate Mouthwash are described in the BP and the PC, respectively. The former wash contains sodium chloride, sodium bicarbonate, concentrated peppermint emulsion and double-strength chloroform water. Extemporaneously compounded preparations include allopurinol at a strength of about 0.1% prepared from tablets in a suspending vehicle of 0.5% methylcellulose sweetened and flavored. Modifications of this preparation have been shown to have considerable stability.

Juices

A juice is prepared from fresh ripe fruit, is aqueous in character and is used in making syrups which are employed as vehicles. The freshly expressed juice is preserved with benzoic acid and allowed to stand at room temperature for several days, until the pectins which naturally are present are destroyed by enzymatic action, as indicated by the filtered juice yielding a clear solution with alcohol. Pectins, if allowed to remain, would cause precipitation in the final syrup.

Cherry Juice (RPS-18 page 1320) is described in the USP XXI and Raspberry Juice in USP XVIII. Concentrated Raspberry Juice PC is prepared from the clarified juice of raspberries. Pectinase is stirred into pulped raspberries and the mixture allowed to stand for 12 hours. The pulp is pressed, the juice clarified and sufficient sucrose added to adjust the weight at 20° to 1.050 to 1.060 g per mL. The juice then is concentrated to one-sixth of its original volume. Sufficient sulfurous acid or sodium metabisulfite is added as a preservative.

Artificial flavors now have replaced many of the natural fruit juices. Although they lack the flavor of the natural juice, they are more stable and easier to incorporate into the final pharmaceutical form. Commercial juices such as orange, apple, grape and mixed vegetables have been used recently to prepare extemporaneous preparations of cholestyramine and nizatidine.

Information on cranberry juice indicates that it may be effective in controlling some urinary tract infections and urolithiasis.

Nasal Solutions

Nasal solutions are usually aqueous solutions designed to be administered to the nasal passages in drops or sprays. While many of the drugs are administered for their local sympathomimetic effect such as Ephedrine Sulfate or Napha-

zoline Hydrochloride Nasal Solution USP, to reduce nasal congestion, a few other official preparations, Lypressin Nasal Solution USP and Oxytocin Nasal Solution USP, are administered in spray form for their systemic effect for the treatment of diabetes insipidus and *milk letdown* prior to breast feeding, respectively. The current route of administration of peptides and proteins is limited to parenteral injection because of inactivation within the gastrointestinal tract. As a result, there is considerable research on intranasal delivery of these drugs such as analogs of enkephalins or luteinizing hormone releasing hormone and insulin. Other drugs which are absorbed poorly from the GI tract such as gentamicin sulfate, are being administered in the form of nasal solutions, in order to obtain appropriate blood levels.

Nasal solutions are prepared so that they are similar in many respects to nasal secretions, so that normal ciliary action is maintained. Thus, the aqueous nasal solutions usually are isotonic and slightly buffered to maintain a pH of 5.5 to 6.5. In addition, antimicrobial preservatives, similar to those used in ophthalmic preparations, and appropriate drug stabilizers, if required, are included in the formulation.

Commercial nasal preparations, in addition to the drugs listed above also include antibiotics, antihistamines and drugs for asthma prophylaxis.

A formula for Ephedrine Nasal Drops PC is

Ephedrine Hydrochloride	0.5 g
Chlorobutanol	0.5 g
Sodium Chloride	0.5 g
Water for preparations	to 100 mL

Current studies indicate that nasal sprays are deposited mainly in the atrium and cleared slowly into the pharynx with the patient in an upright position. Drops spread more extensively than the spray and three drops cover most of the walls of the nasal cavity, with the patient in a supine position and head tilted back and turned left and right. It is suggested that drop delivery, with appropriate movement by the patient, leads to extensive coverage of the walls of the nasal cavity.

Otic Solutions

These solutions occasionally are referred to as aural preparations. Other otic preparations often include formulations such as suspensions and ointments for topical application in the ear.

The main classes of drugs used for topical administration to the ear include analgesics, eg, benzocaine; antibiotics, eg, neomycin; and anti-inflammatory agents, eg, cortisone. The USP preparations include Antipyrine and Benzocaine Otic Solution. The Neomycin and Polymyxin B Sulfates and Hydrocortisone Otic Solutions contain appropriate buffers and dispersants usually in an aqueous solution. The main solvents used in these preparations include glycerin or water. The viscous glycerin vehicle permits the drug to remain in the ear for a long time. Anhydrous glycerin, being hygroscopic, tends to remove moisture from surrounding tissues, thus reducing swelling. Viscous liquids like glycerin or propylene glycol are used either alone or in combination with a surfactant to aid in the removal of cerumen (ear wax). Sodium Bicarbonate Ear-Drops BP may be used if wax is to be removed from the ear. This preparation contains sodium bicarbonate (5 g), glycerin (30 mL) and purified water (a sufficient quantity to make 100 mL).

In order to provide sufficient time for aqueous preparations to act, it is necessary for the patient to remain on his side for a few minutes so the drops do not run out of the ear. Otic preparations are dispensed in a container which permits the administration of drops.

Irrigation Solutions

These solutions are used to wash or bathe surgical incisions, wounds or body tissues. Because they come in contact with exposed tissue, they must meet stringent requirements for injections of the USP such as sterility, particulate

matt.
prod
Water
taine
cont
tion
USP:
Sulfo
and I
and S
Ex
ing a

Sol
muci
semi
to the
(gum

Syrup

Syr
in wa
is use
know
certa
adde
solub
a pre
tance
antir
tains
medi
not r
antly
vehic
and C

Fla
exten
both
little,
that
make
Howe
tered
and
drug
subst
conte
restr

Syr
saline
disgu
This
doub
the li
also
the I
1393
value
medi
flavor
bitter
a suit
dicto
panel
Meer
for fi
and t
of pa
of bu
agen

matter and the requirements of the Pyrogen Test. These products are prepared by dissolving the active ingredient in Water for Injection. They are packaged in single-dose containers, preferably Type I or Type II glass, or suitable plastic containers, and then sterilized. See Chapter 84 for sterilization procedures. A number of irrigations are described in the USP: Acetic Acid Irrigation for bladder irrigation, Dimethyl Sulfoxide Irrigation for relief of internal cystitis, Neomycin and Polymyxin B Sulfates Solution for Irrigation for infection and Sodium Chloride Irrigation for washing wounds.

Extemporaneous formulations frequently are prepared using an isotonic solution of sodium chloride as the solvent.

Sweet or Other Viscid Aqueous Solutions

Solutions which are sweet or viscid include syrups, honeys, mucilages and jellies. All of these are viscous liquids or semisolids. The basic sweet or viscid substances giving body to these preparations are sugars, polyols or polysaccharides (gums).

Syrups

Syrups are concentrated solutions of sugar such as sucrose in water or other aqueous liquid. When Purified Water alone is used in making the solution of sucrose, the preparation is known as *Syrup*, or *simple syrup*. In addition to sucrose, certain other polyols, such as glycerin or sorbitol, may be added to retard crystallization of sucrose or to increase the solubility of added ingredients. Alcohol often is included as a preservative and also as a solvent for flavors; further resistance to microbial attack can be enhanced by incorporating antimicrobial agents. When the aqueous preparation contains some added medicinal substance, the syrup is called a *medicated syrup*. A *flavored syrup* is one which usually is not medicated, but which contains various aromatic or pleasantly flavored substances and is intended to be used as a vehicle or flavor for prescriptions, eg, Acacia, Cherry, Cocoa and Orange USP XXI.

Flavored syrups offer unusual opportunities as vehicles in extemporaneous compounding and are accepted readily by both children and adults. Because they contain no, or very little, alcohol they are vehicles of choice for many of the drugs that are prescribed by pediatricians. Their lack of alcohol makes them superior solvents for water-soluble substances. However, sucrose-based medicines continuously administered to children apparently cause an increase in dental caries and gingivitis; consequently, alternate formulations of the drug either unsweetened or sweetened with noncariogenic substances should be considered. A knowledge of the sugar content of liquid medicines is useful for patients who are on a restricted calorie intake; a list has been prepared by Bergen.¹⁰

Syrups possess remarkable masking properties for bitter or saline drugs. Glycyrrhiza syrup has been recommended for disguising the salty taste of bromides, iodides and chlorides. This has been attributed to its colloidal character and its double sweetness—the immediate sweetness of the sugar and the lingering sweetness of the glycyrrhizin. This syrup is also of value in masking bitterness in preparations containing the B complex vitamins. Acacia Syrup USP XXI (page 1393), because of its colloidal character, is of particular value as a vehicle for masking the disagreeable taste of many medications. Raspberry Syrup BP is one of the most efficient-flavoring agents and is especially useful in masking the taste of bitter drugs. Many factors, however, enter into the choice of a suitable flavoring agent. Literature reports are often contradictory and there appears to be no substitute for the taste panel. The literature on this subject has been reviewed by Meer¹¹ and this reference and Chapter 80 should be consulted for further information on the flavoring of pharmaceuticals and the preparation of a number of official syrups. A series of papers by Schumacher deals with improving the palatability of bulk-compounded products using flavoring and sweetening agents.¹²

For example, cefazolin or gentamicin in 0.9% sodium chloride are used as anti-infective irrigations, dinoprostone in lactated ringers injection is used by continuous intrauterine irrigation for severe postpartum hemorrhage and 5-fluorouracil in 0.9% sodium chloride is employed for bladder irrigation. Alum, either potassium or ammonium, in either sterile water or 0.9% sodium chloride for irrigation has been used for bladder hemorrhage. Amphotericin in sterile water has been used for the treatment of localized infections of the bladder and urinary tract. All the extemporaneous preparations should meet the general requirements noted above for USP irrigations.

In manufacturing syrups the sucrose must be selected carefully and a purified water, free from foreign substances, and clean vessels and containers must be used. The operation must be conducted with care to avoid contamination, if the products are to be stable.

It is important that the concentration of sucrose approach but not quite reach the saturation point. In dilute solutions sucrose provides an excellent nutrient for molds, yeasts and other microorganisms. In concentrations of 65% by weight or more, the solution will retard the growth of such microorganisms. However, a saturated solution may lead to crystallization of a part of the sucrose under conditions of changing temperature.

When heat is used in the preparation of syrups, there is almost certain to be an inversion of a slight portion of the sucrose. Sucrose solutions are dextrorotary but, as hydrolysis proceeds, the optical rotation decreases and becomes negative when the reaction is complete. This reaction is termed *inversion* because *invert sugar* (dextrose plus levulose) is formed. The speed of inversion is increased greatly by the presence of acids; the hydrogen ion acts as a catalyst in this hydrolytic reaction. Invert sugar is more readily fermentable than sucrose and tends to be darker in color. Nevertheless, its two reducing sugars are of value in retarding the oxidation of other substances.

Invert Syrup is described in the BP. It is prepared by hydrolyzing sucrose with hydrochloric acid and neutralizing the solution with calcium or sodium carbonate. The sucrose in the 66.7% w/w solution must be at least 95% inverted. The monograph states that invert syrup, when mixed in suitable proportions with syrup, prevents the deposition of crystals of sucrose under most conditions of storage.

The levulose formed during inversion is sweeter than sucrose and, therefore, the resulting syrup is sweeter than the original syrup. The relative sweetness of levulose, sucrose and dextrose is in the ratio of 173:100:74. Thus, invert sugar is $1/100 (173 + 74)_{1/2} = 1.23$ times as sweet as sucrose. The levulose formed during the hydrolysis also is responsible for the darkening of syrup. It is sensitive to heat and darkens readily, particularly in solution. When syrup or sucrose is overheated, it caramelizes. See *Caramel* (RPS-18 page 1290). Occasionally, it is appropriate to use a sugar-free liquid preparation; a list of these has been published.¹³

Preparation—Syrups are prepared in various ways, the choice of the proper method depends on the physical and chemical characteristics of the substances entering into the preparation.

Solution with Heat—This is the usual method of making syrups when the valuable constituent is neither volatile nor injured by heat, and when it is desirable to make the syrup rapidly. The sucrose usually is added to the purified water or aqueous solution and heated until solution is effected, then strained and sufficient purified water added to make the desired weight or volume. If the syrup is made from an infusion, a decoction or an aqueous solution containing organic matter, such as sap from maple trees, it usually is proper to heat the syrup to the boiling point to coagulate albuminous matter; subsequently, this is separated by straining. If the albumin or other impurities were permitted to remain in the syrup, fermentation probably would be induced in warm weather. Saccharometers are very useful in making syrups by the hot process in cases where the proper

specific gravity of the finished syrup is known. They may be floated in the syrup while boiling, and thus the exact degree of concentration determined without waiting to cool the syrup and having to heat it again to concentrate it further. When taking a reading of the specific gravity of the hot syrup, allowance must be made for the variation from the official temperature (specific gravities in the USP are taken at 25°).

Excessive heating of syrups at the boiling temperature is undesirable since more or less inversion of the sucrose occurs with an increased tendency to ferment. Syrups cannot be sterilized in an autoclave without some caramelization. This is indicated by a yellowish or brownish color resulting from the formation of caramel by the action of heat upon sucrose. The formula and procedure given for Acacia Syrup (page 1393) illustrates this method of preparation.

Agitation without Heat—This process is used in those cases where heat would cause the loss of valuable, volatile constituents. In making quantities up to 2000 mL the sucrose should be added to the aqueous solution in a bottle of about twice the size required for the syrup. This permits active agitation and rapid solution. Stoppering the bottle is important, as it prevents contamination and loss during the process. The bottle should be allowed to lie on its side when not being agitated. Glass-lined tanks with mechanical agitators, especially adapted to dissolving of sucrose, are used for making syrups in large quantities.

This method and that previously described are used for the preparation of a wide variety of preparations that are described popularly as syrups. Most cough syrups, for example, contain sucrose and one or more active ingredients. However, the exact composition of such products is not given on the label. Furthermore, some of these products are listed in the USP but no directions are given for their preparation. For example, Guaifenesin Syrup USP (glyceryl guaiacolate syrup) is official but the only known ingredients are guaifenesin (glyceryl guaiacolate) and ethanol (not less than 3% or more than 4%).

The PC, on the other hand, gives a method for the preparation of Codeine Phosphate Syrup. This contains codeine phosphate (5 g), water for preparations (15 mL), chloroform spirit (25 mL) and sufficient syrup to make 1000 mL. It can be used for the relief of cough. Another syrup for this purpose is Codeine Linctus PC. This is really a medicated syrup which possesses demulcent, expectorant or sedative properties. Unlike the syrup, it is colored and flavored. The formula for Codeine Linctus PC is

Codeine Phosphate	3 g
Compound Tartrazine Solution	10 mL
Benzoic Acid Solution	20 mL
Chloroform Spirit	20 mL
Water for Preparations	20 mL
Lemon Syrup	200 mL
Syrup	to 1000 mL

Dissolve the codeine phosphate in the water, add 500 mL of the syrup and mix. Add the other ingredients and sufficient syrup to produce 1000 mL.

For pediatric use, 200 mL of this linctus is diluted with sufficient syrup to make 1000 mL. If sugar is contraindicated in the diet, Diabetic Codeine Linctus can be used:

Codeine Phosphate	3 g
Citric Acid monohydrate	5 g
Lemon Spirit	1 mL
Compound Tartrazine Solution	10 mL
Benzoic Acid Solution	20 mL
Chloroform Spirit	20 mL
Water for Preparations	20 mL
Sorbitol Solution	to 1000 mL

Dissolve the codeine phosphate and the citric acid in the water, add 750 mL of the sorbitol solution and mix. Add the other ingredients and sufficient sorbitol solution to produce 1000 mL.

Sorbitol Solution is the sweetening agent and contains 70% w/w of total solids, consisting mainly of D-sorbitol. It has about half the sweetening power of syrup. In the US the FDA has banned the use of chloroform in medicines and cosmetics because of reported carcinogenicity in animals.

Basic formulations can be varied easily to produce the highly advertised articles of commerce. The prescription-only drug (eg, codeine phosphate or methadone) must, of course, be omitted from the formulation but, in certain countries, such as Canada, a decreased quantity of codeine phosphate is permitted in an OTC cough syrup. In addition to the ingredients cited or listed in the official compendia (eg, tolu, squill or ipecacuanha), many cough syrups contain an antihistamine.

Many other active ingredients (eg, ephedrine sulfate, dicyclomine hydrochloride, chloral hydrate or chlorpromazine hydrochloride) are marketed as syrups. Like cough syrups, these preparations are flavored, colored

and recommended in those instances where the patient cannot swallow the solid dosage form.

Addition of a Medicating Liquid to Syrup—This method is resorted to in those cases in which fluid extracts, tinctures or other liquids are added to syrup to medicate it. Syrups made in this way usually develop precipitates since alcohol is often an ingredient of the liquids thus used, and the resinous and oily substances dissolved by the alcohol precipitate when mixed with the syrup, producing unsightly preparations. A modification of this process, frequently adopted, consists of mixing the fluid extract or tincture with the water, allowing the mixture to stand to permit the separation of insoluble constituents, filtering and then dissolving the sucrose in the filtrate. It is obvious that this procedure is not permissible when the precipitated ingredients are the valuable medicinal agents.

The formula and procedure given for Aromatic Eriodictyon Syrup USP XXI (RPS-18 page 1301) illustrate this method of preparation.

Percolation—In this procedure, purified water, or an aqueous solution, is permitted to pass slowly through a bed of crystalline sucrose, thus dissolving it and forming a syrup. A cotton pledget is placed in the neck of the percolator and the water or aqueous solution added. By means of a suitable stopcock the flow is regulated so that drops appear in rapid succession. If necessary, a portion of the liquid is recycled through the percolator to dissolve all the sucrose. Finally, sufficient purified water is passed through the cotton to make the required volume.

To be successful in using this process, care in several particulars must be exercised: (1) the percolator used should be cylindrical or semicylindrical and cone-shaped as it nears the lower orifice; (2) a coarse granular sugar must be used, otherwise it will coalesce into a compact mass, which the liquid cannot permeate; (3) the purified cotton must be introduced with care.

If pressed in too tightly, the cotton will stop the process effectually; if inserted too loosely, the liquid will pass through the cotton rapidly and the filtrate will be weak and turbid (from imperfect filtration); it should be inserted completely within the neck of the percolator, since a protruding end, inside the percolator, up through the sucrose, will permit the last portions of water to pass out at the lower orifice without dissolving all the sucrose. For specific directions see *Syrups* (page 1393). The process of percolation is applied on a commercial scale for the making of official syrups as well as those for confectionary use.

Percolation is the preferred method for the preparation of Syrup USP (page 1301). The sucrose, in this instance, is placed in the percolator. However, a slightly modified approach must be used if a drug of vegetable origin is to be incorporated into the syrup. For example, wild cherry bark is first percolated with water; the collection vessel contains sucrose (800 g) and glycerol (50 mL). When the total volume is 1000 mL, the percolate is agitated to produce Wild Cherry Syrup PC.

Reconstitution—In order to improve stability and minimize microbial contamination, dry syrup formulations can be prepared and Purified Water USP added just prior to dispensing or use. Powder mixtures, wholly granulated products and partially granulated products have been investigated for this purpose by Ryder.¹⁴

The powder mixture preparation requires less equipment and energy to prepare. Chemical stability problems are minimal, since no heat or solvents are used in the process and a low moisture content can be obtained in the final product; unfortunately, powder mixtures are prone to homogeneity problems. In the case of the wholly granulated product all the ingredients are included in the granulation stage. The drug may be incorporated into the dry product before granulation or dissolved or suspended in the granulating fluid. After formation, the granules are dried and then screened to break down oversize particles. The advantages of granulated over powder mixtures include better appearance, better flow, fewer segregation problems and less dust during processing. Partially granulated mixtures are used to gain some of the advantages of granulation without the disadvantages. Usually the drug, and other fine particles, are included at the granulation stage, perhaps with some diluents to improve flow and reduce segregation and dust. Materials selected for mixing with the dried granules would include thermolabile excipients, such as flavors, and free flowing materials, such as sugars.

Preservation—Syrups should be made in quantities which can be consumed within a few months, except in those cases where special facilities can be employed for their preservation; a low temperature is the best method. Concentration without super-saturation is also a condition favorable to preservation. The USP states that syrups may contain preservatives such as glycerin, methylparaben, benzoic acid and sodium benzoate to prevent bacterial and mold growth. Combinations of alkyl esters of p-hydroxybenzoic acid are effective inhibitors of yeasts which have been implicated in the contamination of commercial syrups.

The official syrups should be preserved in well-dried bottles, preferably those which have been sterilized. These

bottle
durin
pered

Bla
pared
One v
11 vo
a sim
rants,
destr
ing 7
prese
is per
ment
cherry
(page
Syr

for pr
drug
not re
excipi
water
the of
insolu
some
above
only i
ity an
pared
midaz
amide
appro
When
sion
solubl
tions
cefure
tone,
pensi
that a
distrib
: chapt
as val
ups, v
a unifi

Hone

Hon

syrup
base.
but at
most
BP lis
Oxym
prepa
Hoi

been
applic
absce
sugar
polye
conce
benef

Muci

The
produ
mucil

bottles should not hold more than is likely to be required during 4 to 6 weeks and should be filled completely, stoppered carefully and stored in a cool, dark place.

Syrups Prepared from Juices

Blackberry, pineapple and strawberry syrups may be prepared by following the directions for Raspberry Syrup BP. One volume of the concentrated raspberry juice is diluted with 11 volumes of syrup. Black Current Syrup BP is prepared in a similar manner but also can be prepared from black currants, with certain modifications. The pectin in the juice is destroyed with pectinase. The syrup is prepared by dissolving 700 g of sucrose in 560 mL of clarified juice and may be preserved with sodium metabisulfite. The addition of a dye is permitted, provided it complies with the pertinent government regulations. Cherry Syrup USP XXI is prepared from cherry juice by the addition of alcohol, sucrose and water (page 1393).

Syrups, either as a syrup or as a flavored syrup, are useful for preparing liquid oral dosage forms from not only the pure drug but also injections, capsules or tablets if the pure drug is not readily available. On one hand, if the drug and all the excipients in the preparation, eg, injectables or capsules, are water soluble, a solution will result if a syrup is prepared. On the other hand, if the preparation to be used contains water-insoluble ingredients, as is usually the case with tablets and some capsules, a suspension will be formed. Several of the above preparations have been described in the literature, not only in regard to their formulation but also in regard to stability and bioavailability. Some drugs which have been prepared from either the pure drug or an injectable form include midazolam, atropine, aminocaproic acid, terbutaline, procainamide, chloroquin, propranolol and citrated caffeine. If the appropriate salt of the drug is used, a solution will result. When tablets are used to prepare liquid formulations, a suspension usually is formed because there is usually a nonwater-soluble ingredient used in tablet preparations. Some formulations prepared from tablets are clonidine hydrochloride, cefuroxime axetil, famotidine, terbutaline sulfate, spironolactone, ranitidine, propranolol and rifampin. The resulting suspensions should have a uniform distribution of particles so that a consistent dose is obtained. If the materials are not distributed uniformly, more appropriate suspending formulations should be considered, which are described later in the chapter. If pharmaceutical preparations contain a liquid such as valproic acid or simethicone, to be incorporated into syrups, which is insoluble in water, an emulsion will form and a uniform product will not result.

Honeys

Honeys are thick liquid preparations somewhat allied to the syrups, differing in that honey, instead of syrup, is used as a base. They are unimportant as a class of preparations today but at one time, before sugar was available and honey was the most common sweetening agent, they were used widely. The BP lists one preparation for coughs containing honey. Squill Oxymel contains squill, water, acetic acid and honey and is prepared by a maceration process.

Honey and sugar pastes are used to a small extent and have been discussed in the pharmaceutical literature for topical application for the treatment of certain types of ulcers and abscesses. Thick and thin sugar pastes containing Caster sugar (very fine granular sugar), icing sugar (additive-free), polyethylene glycol 400 and hydrogen peroxide (in a final concentration of 0.15%) have been prepared and shown to be beneficial in the process of wound healing.

Mucilages

The official mucilages are thick, viscid, adhesive liquids, produced by dispersing gum in water, or by extracting the mucilaginous principles from vegetable substances with water.

The mucilages all are prone to decomposition, showing appreciable decrease in viscosity on storage; they should never be made in quantities larger than can be used immediately, unless a preservative is added. Acacia Mucilage NF XII contains benzoic acid and Tragacanth Mucilage BPC (1973) contains alcohol and chloroform water. Chloroform in manufactured products for internal use is banned in some countries.

Acacia Mucilage may be prepared by placing 350 g of acacia in a graduated bottle, washing the drug with cold purified water, allowing it to drain and adding enough warm purified water, in which 2 g of benzoic acid has been dissolved, to make the product measure 1000 mL. The bottle then is stoppered, placed on its side, rotated occasionally and the product strained when the acacia has dissolved.

Tragacanth Mucilage BPC (1973) is prepared by mixing 12.5 g of tragacanth with 25 mL alcohol (90%) in a dry bottle and then quickly adding sufficient chloroform water to 1000 mL and shaking vigorously. The alcohol is used to disperse the gum to prevent agglomeration on addition of the water.

Mucilages are used primarily to aid in suspending insoluble substances in liquids; their colloidal character and viscosity help prevent immediate sedimentation. Examples include sulfur in lotions, resin in mixtures and oils in emulsions. Both tragacanth and acacia either are partially or completely insoluble in alcohol. Tragacanth is precipitated from solution by alcohol, but acacia, on the other hand, is soluble in diluted alcoholic solutions. A 60% solution of acacia may be prepared with 20% alcohol and a 4% solution of acacia may be prepared even with 50% alcohol.

The viscosity of tragacanth mucilage is reduced by acid, alkali or sodium chloride, particularly if the mucilage is heated. It shows maximum viscosity at pH 5. Acacia is hydrolyzed by dilute mineral acids to arabinose, galactose, aldobionic and galacturonic acids. Its viscosity is low but is maintained over a wide pH range.

Recent research on mucilages includes the preparation of mucilage from plantain and the identification of its sugars, the preparation and suspending properties of cocoa gum, the preparation of glycerin ointments using flaxseed mucilage and the consideration of various gums and mucilages obtained from several Indian plants for pharmaceutical purposes.

Several synthetic mucilage-like substances such as *polyvinyl alcohol*, *methylcellulose*, *carboxymethylcellulose* and related substances, as described in Chapter 80, are used at the appropriate concentration as mucilage substitutes, emulsifying and suspending agents. Methylcellulose (page 1397) is used widely as a bulk laxative since it absorbs water and swells to a hydrogel in the intestine, in much the same manner as *psyllium* or *karaya gum*. Methylcellulose Oral Solution USP is a flavored solution of the agent. It may be prepared by adding slowly the methylcellulose to about one-third the amount of boiling water, with stirring, until it is thoroughly wetted. Cold water then should be added and the wetted material allowed to dissolve while stirring. The viscosity of the solution will depend upon the concentration and the specifications of the methylcellulose. The synthetic gums are non-glycogenetic and may be used in the preparation of diabetic syrups. Several formulas for such syrups, based on sodium carboxymethylcellulose, have been proposed.

Uniformly smooth mucilages sometimes are difficult to prepare due to the uneven wetting of the gums. In general, it is best to use fine gum particles and disperse them with good agitation in a little 95% alcohol or in cold water (except for methylcellulose). The appropriate amount of water then can be added with constant stirring. A review of the chemistry and properties of acacia and other gums has been prepared.¹⁵

Jellies

Jellies are a class of gels in which the structural coherent matrix contains a high portion of liquid, usually water. They are similar to mucilages, in that they may be prepared from

similar gums, but they differ from the latter in having a jelly-like consistency. A whole gum of the best quality, rather than a powdered gum, is desirable in order to obtain a clear preparation of uniform consistency. Tragacanth is the gum used in the preparation of Ephedrine Sulfate Jelly NF XII. While the specific thickening agent in the USP jellies is not indicated, reference usually is made in the monograph to a water-soluble, sterile, viscous base. These preparations also may be formulated with water from acacia, chondrus, gelatin, carboxymethylcellulose, hydroxyethylcellulose and similar substances.

Jellies are used as lubricants for surgical gloves, catheters and rectal thermometers. Lidocaine Hydrochloride Jelly USP

is used as a topical anesthetic. Therapeutic vaginal jellies are available and certain jelly-like preparations are used for contraceptive purposes, which often contain surface-active agents to enhance the spermaticidal properties of the jelly. Aromatics, such as methyl salicylate and eucalyptol, often are added to give the preparation a desirable odor.

Jellies are prone to microbial contamination and therefore contain preservatives, eg, methyl *p*-hydroxybenzoate is used as a preservative in a base for medicated jellies. This base contains sodium alginate, glycerin, calcium gluconate and water. The calcium ions cause a cross-linking with sodium alginate to form a gel of firmer consistency. A discussion of gels is provided later in the chapter.

Nonaqueous Solutions

It is difficult to evaluate fairly the importance of nonaqueous solvents in pharmaceutical processes. That they are important in the manufacture of pharmaceuticals is an understatement. However, pharmaceutical preparations, and, in particular, those intended for internal use, rarely contain more than minor quantities of the organic solvents that are common to the manufacturing or analytical operation. For example, industry uses large quantities of chloroform in some operations but the solvent is of only minor importance with respect to the final product. One mL of chloroform dissolves in about 200 mL of water and the solution so formed finds some use as a vehicle (see the section on *Aromatic Waters*). Chloroform has been an ingredient in a number of cough syrups in the past but it has been banned in the US by the FDA in manufactured products intended for internal use. Solvents such as acetone, benzene and petroleum ether must not be ingredients in preparations intended for internal use.

Products of commerce for internal use may contain solvents such as ethanol, glycerin, propylene glycol, certain oils and liquid paraffin. Preparations intended for external use may contain solvents in addition to those just mentioned, namely isopropyl alcohol, polyethylene glycols, various ethers and certain esters. A good example of preparations of this type are the rubefacient rubbing alcohols. Rubbing Alcohol must be manufactured in accordance with the requirements of the Bureau of Alcohol, Tobacco and Firearms, US Treasury Dept, using Formula 23-H denatured alcohol. This mixture contains 8 parts by volume of acetone, 1.5 parts by volume of methyl isobutyl ketone and 100 parts by volume of ethanol. Besides the alcohol in the Rubbing Alcohol, the final product must contain water, sucrose octaacetate or denatonium benzoate and may contain color additives, perfume oils and a suitable stabilizer. The alcohol content, by volume, is not less than 68.5% and not more than 71.5%. The isopropyl alcohol content in Isopropyl Rubbing Alcohol can vary from 68.0% to 72.0% and the finished product may contain color additives, perfume oils and suitable stabilizers.

Although the lines between aqueous and nonaqueous preparations tend to blur in those cases where the solvent is water-soluble, it is possible to categorize a number of products as nonaqueous. This section is, therefore, devoted to groups of nonaqueous solutions; the alcoholic or hydroalcoholic solutions (eg, elixirs and spirits), ethereal solutions (eg, collodions), glycerin solutions (eg, glycerins), oleaginous solutions (eg, liniments, oleovitamins and toothache drops), inhalations and inhalants.

Although the above list is limited, a wide variety of solvents are used in various pharmaceutical preparations. Solvents such as glycerol formal, dimethylacetamide and glycerol dimethylketal have been suggested for some products produced by the industry. However, the toxicity of many of these solvents is not well-established and, for this reason, careful clinical studies should be carried out on the formulated product before it is released to the marketplace.

It is essential that the toxicity of solvents be tested appropriately and approved in order to avoid problems: for example,

the tragic loss of life which occurred during 1937 when diethylene glycol was used in an elixir of sulfanilamide. The result of this tragedy was the 1938 Federal Food, Drug and Cosmetic Act, which required that products be tested for both safety and effectiveness.

Collodions

Collodions are liquid preparations containing pyroxylin (a nitrocellulose) in a mixture of ethyl ether and ethanol. They are applied to the skin by means of a soft brush or other suitable applicator and, when the ether and ethanol have evaporated, leave a film of pyroxylin on the surface. The official medicated collodion, Salicylic Acid Collodion USP, contains 10% *w/v* of salicylic acid in Flexible Collodion USP and is used as a keratolytic agent in the treatment of corns and warts. Collodion USP and Flexible Collodion USP are water-repellent protectives for minor cuts and scratches. Collodion is made flexible by the addition of castor oil and camphor. Collodion has been used to reduce or eliminate the side effects of fluorouracil treatment of solar keratoses. Vehicles other than flexible collodion, such as a polyacrylic base, have been used to incorporate salicylic acid for the treatment of warts with less irritation.

Elixirs

Elixirs are clear, pleasantly flavored, sweetened hydroalcoholic liquids intended for oral use. The main ingredients in elixirs are ethanol and water but glycerin, sorbitol, propylene glycol, flavoring agents, preservatives and syrups often are used in the preparation of the final product. Elixirs are more fluid than syrups, due to the use of less viscous ingredients such as alcohol and the minimal use of viscosity-improving agents such as sucrose. They are used as flavors and vehicles such as Aromatic Elixir USP (page 1394) for drug substances and, when such substances are incorporated into the specified solvents, they are classified as medicated elixirs, eg, Dexamethasone Elixir USP and Phenobarbital Elixir USP. Occasionally, certain adverse effects, eg, mucosal erosions, may be eliminated or reduced if the active drug, eg, potassium chloride, is administered in elixir rather than in a solid dosage form.

The distinction between some of the medicated syrups and elixirs is not always clear. For example, Ephedrine Sulfate Syrup USP contains between 20 and 40 mL of alcohol in 1000 mL of product. Ephedrine Elixir BP contains a suitable flavored vehicle and 12% alcohol. Definitions are, sometimes, inconsistent and, in some instances, not too important with respect to the naming of the articles of commerce. To be designated as an elixir, however, the solution must contain alcohol.

The alcoholic content will vary greatly, from elixirs containing only a small quantity to those that contain a considerable portion as a necessary aid to solubility. For example, Aro-

mati
Benz
El
adde
sweet
fects
Clair
tute
terpi
disa
tions
elixa
Or
give

Te
Or
Be
So
Pr
Al
Pu

Dis
whic
alcoh
purifi

Th
mal
mas
Al
does
It of
vent
the
ation
hydr
Be
to be
ture
amo
alco
proc

Dis
solu
solu
alcoh

St
prop
tion
tain
alco
The
the
O
(RP
tion
ing
mer
For
with
veg
gate
stre
be
usu
pre
holi
be
T

C
Si
G
A

matic Elixir USP contains 21 to 23% of alcohol; Compound Benzaldehyde Elixir USP, on the other hand, contains 3 to 5%.

Elixirs also may contain glycerin and syrup. These may be added to increase the solubility of the medicinal agent for sweetening purposes or to decrease the pharmacological effects of the alcohol. Some elixirs contain propylene glycol. Claims have been made for this solvent as a satisfactory substitute for both glycerin and alcohol. Sumner,¹⁶ in his paper on terpin hydrate preparations, summarized the advantages and disadvantages of this solvent and suggested several formulations with therapeutic characteristics superior to those of the elixir described in NF XIII.

One of the four formulations described in Sumner's paper is given below:

Terpin Hydrate.....	6.0 g
Orange Oil.....	0.1 mL
Benzaldehyde.....	0.005 mL
Sorbitol Solution USP.....	10.0 mL
Propylene Glycol.....	40.0 mL
Alcohol.....	43.0 mL
Purified Water, a sufficient quantity to make.....	100.0 mL

Dissolve the terpin hydrate in the propylene glycol and sorbitol solution which have been heated to 50°. Add the oil and the benzaldehyde to the alcohol and mix with the terpin hydrate solution at 25°. Add sufficient purified water to make the product measure 100 mL.

The elixir contains 300 mg of terpin hydrate/5 mL, a minimal quantity of alcohol and flavoring agents which adequately mask the taste of propylene glycol.

Although alcohol is an excellent solvent for some drugs, it does accentuate the saline taste of bromides and similar salts. It often is desirable, therefore, to substitute some other solvent that is more effective in masking such tastes for part of the alcohol in the formula. In general, if taste is a consideration, the formulator is more prone to use a syrup rather than a hydroalcoholic vehicle.

Because only relatively small quantities of ingredients have to be dissolved, elixirs are more readily prepared and manufactured than syrups, which frequently contain considerable amounts of sugar. An elixir may contain both water- and alcohol-soluble ingredients. If such is the case, the following procedure is indicated:

Dissolve the water-soluble ingredients in part of the water. Add and solubilize the sucrose in the aqueous solution. Prepare an alcoholic solution containing the other ingredients. Add the aqueous phase to the alcoholic solution, filter and make to volume with water.

Sucrose increases viscosity and decreases the solubilizing properties of water and so must be added after primary solution has been effected. A high alcoholic content is maintained during preparation by adding the aqueous phase to the alcoholic solution. Elixirs always should be brilliantly clear. They may be strained or filtered and, if necessary, subjected to the clarifying action of purified talc or siliceous earth.

One of the former official elixirs, Iso-Alcoholic Elixir NF XV (RPS-18 page 1328), actually is a combination of two solutions, one containing 8 to 10% alcohol and the other containing 73 to 78%. It is used as a vehicle for various medicaments that require solvents of different alcoholic strengths. For example, the alcoholic strength of the elixir to be used with a single liquid galenical, which is a liquid preparation of vegetable origin, is approximately the same as that of the galenical. When preparations with different alcoholic strengths are employed in the same prescription, the elixir to be used is the one that produces the best solution. This is usually the average of the alcoholic strengths of the several preparations. For nonextractive substances, the lowest alcoholic strength of elixir that will produce a clear solution should be selected.

The formula for High-Alcoholic Elixir is

Compound Orange Spirit.....	4 mL
Saccharine.....	3 g
Glycerin.....	200 mL
Alcohol, a sufficient quantity to make.....	1000 mL

This elixir, and many other liquid preparations intended for internal use eg, the diabetic syrups thickened with sodium carboxymethylcellulose or similar substances, contain saccharin as a sweetening agent. In the past, scientists have studied the toxic effects of this sweetening agent and found bladder tumors in rats. However, it is now generally accepted that this does not apply to humans when saccharin is used as a sweetener. Research on another sweetening agent, cyclamate,¹⁷ showed that it could produce cancer in animals and, as a result, this substance was removed from a wide variety of products.

Cyclamates and saccharin have been banned in some countries as ingredients in manufactured products. Much research has been done to find a safe synthetic substitute for sucrose. As a result, aspartame (methyl *N*-(*L*-α-aspartyl)-*L*-phenylalaninate), which is about 200 times sweeter than sucrose, is being used now in many commercial preparations as the sweetening agent. It is sparingly soluble in water and is most stable at a pH of 4.3.

Incompatibilities—Since elixirs contain alcohol, incompatibilities of this solvent are an important consideration during formulation. Alcohol precipitates tragacanth, acacia and agar from aqueous solutions. Similarly, it will precipitate many inorganic salts from similar solutions. The implication here is that such substances should be absent from the aqueous phase or present in such concentrations that there is no danger of precipitation on standing.

If an aqueous solution is added to an elixir, a partial precipitation of alcohol-soluble ingredients may occur. This is due to the reduced alcoholic content of the final preparation. Usually, however, the alcoholic content of the mixture is not sufficiently decreased to cause separation. As vehicles for tinctures and fluidextracts, the elixirs generally cause a separation of extractive matter from these products due to a reduction of the alcoholic content.

Many of the incompatibilities between elixirs, and the substances combined with them, are due to the chemical characteristics of the elixir *per se*, or of the ingredients in the final preparation. Thus, certain elixirs are acid in reaction while others may be alkaline and will, therefore, behave accordingly.

Glycerins

Glycerins or glycerites are solutions or mixtures of medicinal substances in not less than 50% by weight of glycerin. Most of the glycerins are extremely viscous and some are of a jelly-like consistency. Few of them are used extensively. Glycerin is a valuable pharmaceutical solvent forming permanent and concentrated solutions not otherwise obtainable.

Glycerin is used as the sole solvent for the preparation of Antipyrine and Benzocaine Otic Solution USP. As noted under *Otic Solutions*, glycerin alone is used to aid in the removal of cerumen. Externol, a commercial product, contains 5% carbamide peroxide (urea hydrogen peroxide) in glycerin, has shown superior qualities in dispersing ear wax. A glycerin base was chosen as the optimum solvent for an otic preparation in a study involving the stability and antimicrobial activity of kanamycin sulfate otic drops.

Glycerins are hygroscopic and should be stored in tightly closed containers.

Inhalations and Inhalants

Inhalations

These preparations are so used or designed that the drug is carried into the respiratory tree of the patient. The vapor or mist reaches the affected area and gives prompt relief from the symptoms of bronchial and nasal congestion. The USP defines Inhalations in the following way:

Inhalations are drugs or solutions or suspensions of one or more drug substances administered by the nasal or oral respiratory route for either a local or systemic effect. Solutions of drug substances in sterile water for

inhalation or in sodium chloride inhalation solution may be nebulized by the use of inert gases. Nebulizers are suitable for the administration of inhalation solutions only if they give droplets sufficiently fine and uniform in size so that the mist reaches the bronchioles. Nebulized solutions may be breathed directly from the nebulizer, or the nebulizer may be attached to a plastic face mask, tent or intermittent positive pressure breathing (IPPB) machine.

Another group of products, also known as metered dose inhalers (MDIs) are propellant-driven drug suspensions or solution in liquefied-gas propellant with or without a cosolvent and are intended for delivering metered doses of the drug to the respiratory tract. An MDI contains multiple doses, often exceeding several hundred. The most common single-dose volumes delivered are from 25 to 100 μL (also expressed as mg) per actuation.

Examples of MDIs containing drug solutions and suspension in this pharmacopeia are Epinephrine Inhalation Aerosol and Isoproterenol Hydrochloride and Phenylephrine Bitartrate Inhalation Aerosol, respectively.

Powders also may be administered by mechanical devices that require manually produced pressure or a deep inhalation by the patient, eg, Cromolyn Sodium for Inhalation.

As stated in the USP, particle size is of major importance in the administration of this type of preparation. The various mechanical devices that are used in conjunction with inhalations are described in some detail in Chapter 107. It has been reported that the optimum particle size for penetration into the pulmonary cavity is of the order of 0.5 to 7 μm . Fine mists are produced by pressurized aerosols and hence possess basic advantages over the older nebulizers; in addition, metered aerosols deliver more uniform doses. See Chapter 95. A number of inhalations are described in the USP XXI, eg, Epinephrine Inhalation Solution is a solution of Epinephrine in Purified Water prepared with the aid of Hydrochloric Acid, and Isoproterenol Inhalation Solution is a solution of Isoproterenol Hydrochloride in Purified Water and may contain Sodium Chloride.

The term *inhalations*, defined by the BP, has a different meaning. These are solutions or suspensions of one or more active ingredients which may contain an inert, suspended diffusing agent. They are intended to release volatile constituents for inhalation, either when placed on a pad or when added to hot, but not boiling, water. Benzoin Inhalation BP contains benzoin, storax and alcohol. The vapors from a preparation containing 1 teaspoonful of the tincture and 1 qt of boiling water may be inhaled. The device known as a *vaporizer* may be used with a number of commercially available preparations of this type (see Chapter 107).

Inhalants

The USP defines inhalants as follows:

A special class of inhalations termed "inhalants" consists of drugs or combinations of drugs that, by virtue of their high vapor pressure, can be carried by an air current into the nasal passage where they exert their effect. The container from which the inhalant is administered is known as an inhaler.

Propylhexedrine Inhalant USP and Tuaminoheptane Inhalant USP consist of cylindrical rolls of suitable fibrous material impregnated with propylhexedrine or tuaminoheptane (as carbonate), usually aromatized, and contained in a suitable inhaler. Propylhexedrine is the active ingredient in the widely used Benzedrex Inhaler. Both of these drugs are vasoconstrictors used to relieve nasal congestion. Inhalers which come in contact with the mouth or nasal passages become contaminated by bacteria, thus, they should be restricted to personal use.

Another inhalant is Amyl Nitrite USP which is very flammable and should not be used where it may be ignited. It is packaged in sealed glass vials in a protective gauze. Upon breaking the vial, the gauze absorbs the drug which is then inhaled for the treatment of anginal pain. See page 953.

Liniments

Liniments are solutions or mixtures of various substances in oil, alcoholic solutions of soap or emulsions and may contain suitable antimicrobial preservatives. They are intended

for external application and should be so labeled. They are rubbed onto the affected area and, because of this, were once called *embrocations*.

Liniments usually are applied with friction and rubbing of the skin, the oil or soap base providing for ease of application and massage. Alcoholic liniments are used generally for their rubefacient, counterirritant, mildly astringent and penetrating effects. Such liniments penetrate the skin more readily than do those with an oil base. The oily liniments, therefore, are milder in their action but are more useful when massage is required. Depending on their ingredients, such liniments may function solely as protective coatings. Liniments should not be applied to skin that is bruised or broken.

Many of the marketed "white" liniments are based on the formulation below or variations thereof.

White Liniment BP

Oleic Acid	85 mL
Turpentine Oil	250 mL
Dilute Ammonia Solution	45 mL
Ammonium Chloride	12.5 mL
Purified Water	625 mL

Mix the oleic acid with the turpentine oil. Dilute the dilute ammonia solution with 45 mL of the water, previously warmed, add to the oily solution and shake to form an emulsion. Separately dissolve the ammonium chloride in the remainder of the water, add to the emulsion and mix.

Other liniments contain antipruritics, astringents, emollients or analgesics and are classified on the basis of their active ingredient. An example is:

Compound Calamine Application PC (Compound Calamine Liniment)

Calamine	100 g
Zinc Oxide	50 g
Wool Fat	25 g
Zinc Stearate	25 g
Yellow Soft Paraffin	250 g
Liquid Paraffin	550 g

The powders are triturated to a smooth paste with some of the liquid paraffin (Liquid Petrolatum). The wool fat, zinc stearate and yellow soft paraffin (Petrolatum) are melted, mixed with some of the liquid paraffin, the mixture incorporated with the triturated powders and the rest of the liquid paraffin added with mixing.

Dermatologists prescribe products of this type but only those containing the rubefacients are advertised extensively and used by consumers for treating minor muscular aches and pains. It is essential that these applications be marked clearly for external use only.

Because of the confusion of camphorated oil (camphor liniment) with castor oil, which has resulted in ingestion and, perhaps, to poisoning, camphorated oil has been banned from the market. Camphorated Oil presently is classified as a new drug by the FDA for which a new drug application is required.

Oleovitamins

Oleovitamins are fish-liver oils diluted with edible vegetable oil or solutions of the indicated vitamins or vitamin concentrates (usually vitamin A and D) in fish-liver oil. The definition is broad enough to include a wide variety of marketed products.

Oleovitamin A and D is official; vitamin D may be present as ergocalciferol or cholecalciferol obtained by the activation of ergosterol or 7-dehydrocholesterol or may be obtained from natural sources. Synthetic vitamin A, or a concentrate, may be used to prepare oleovitamin A. The starting material for the concentrate is a fish-liver oil, the active ingredient being isolated by molecular distillation or by a saponification and extraction procedure. The latter procedure is described in detail in the monograph for Concentrated Vitamin A Solution PC.

These vitamins are unstable in the presence of rancid oils and, therefore, these preparations and, in particular, Oleovita-

mir
und
tect

Spi

S
hyd
mat
liqu
tior
(Sp
end
defi
peu
offi

S
valu
flav
reac
For
the
that

T
am
ent
Soa
der:
unb
S
and
volz
to l
alcc
an c

ratio
Si
are
proc

A
St
L
L
N
Al
Pi

D
sol
allo
the
gra
pur
the
agit

Ti
the
cont
cot:
the:

A
two
thro
ete:
par

min A, should be stored in small, tight containers, preferably under vacuum or under an atmosphere of an inert gas, protected from light.

Spirits

Spirits, sometimes known as essences, are alcoholic or hydroalcoholic solutions of volatile substances. Like the aromatic waters, the active ingredient in the spirit may be a solid, liquid or gas. The genealogical tree for this class of preparations begins with the distinguished pair of products, Brandy (*Spiritus Vini Vitis*) and Whisky (*Spiritus Frumenti*), and ends with a wide variety of products that comply with the definition given above. Physicians have debated the therapeutic value of the former products and these are no longer official in the compendia.

Some of these spirits are used internally for their medicinal value, a few medicinally by inhalation and a large number as flavoring agents. The latter group provides a convenient and ready means of obtaining the volatile oil in the proper quantity. For example, a spirit or spirit-like preparation may be used in the formulation of aromatic waters or other pharmaceuticals that require a distinctive flavor.

The BP's definition of Spirits is very broad. Some examples are Aromatic Ammonia Spirits BP, which has a different formula from the USP XXI, is used as a flavoring agent, Soap Spirits BP is used instead of a shampoo for scalp disorders and Surgical Spirits BP is used for its astringent action on unbroken skin.

Spirits should be stored in tight, light-resistant containers and in a cool place. This tends to prevent evaporation and volatilization of either the alcohol or the active principle and to limit oxidative changes. Spirits usually contain a high alcohol content and consequently should be kept away from an open flame.

Preparation—There are four classic methods of preparation:

Simple Solution—This is the method by which the majority of spirits are prepared. Aromatic Ammonia Spirit USP is official and a formula and procedure is given in USP XXI, which illustrates this method of preparation.

Aromatic Ammonia Spirit USP XXI

Ammonium Carbonate, in translucent pieces.....	34 g
Strong Ammonium Solution.....	36 mL
Lemon Oil.....	10 mL
Lavender Oil.....	1 mL
Nutmeg Oil.....	1 mL
Alcohol.....	700 mL
Purified Water, a sufficient quantity to make.....	1000 mL

Dissolve the ammonium carbonate in the strong ammonia solution and 195 mL of purified water by gentle agitation and allow the solution to stand for 12 hours. Dissolve the oils in the alcohol, contained in a graduated bottle or cylinder, and gradually add the ammonium carbonate solution and enough purified water to make the product measure 1000 mL. Set the mixture aside in a cool place for 24 hours, occasionally agitating it, then filter, using a covered funnel.

The spirit is a respiratory stimulant and is administered by inhalation of the vapor as required. It is marketed in suitable tight, light-resistant containers but is also available in a single-dose glass vial wrapped in a soft cotton envelope. The vial is broken easily; the cotton acts as a sponge for the spirit.

Ammonium carbonate is a mixture of ammonium bicarbonate and ammonium carbamate ($\text{NH}_2\text{COONH}_4$). The carbamate reacts with water to form the carbonate. An ammonium carbonate solution is, therefore, a solution of ammonium bicarbonate and ammonium carbonate in water. However, it decomposes in water, the decomposition products being ammonia, carbon dioxide and water. The stability of the spirit is improved by the addition of strong ammonia solution. This represses the hydrolysis of ammonium carbonate and, in this way, decreases the loss of dissolved gases.

Solution with Maceration—In this procedure, the leaves of a drug are macerated in purified water to extract water-soluble matter. They are expressed and the moist, macerated leaves are added to a prescribed quantity of alcohol. The volatile oil is added to the filtered liquid. Peppermint Spirit USP is made by this process (RPS-18 page 798). Peppermint Spirit BP differs from the official product in that it is a solution of the volatile oil in ethanol 90% only. The concentration of volatile oil in the final product is about the same but the official preparation possesses a green color.

Chemical Reaction—No official spirits are prepared by this process. Ethyl nitrite is made by the action of sodium nitrite on a mixture of alcohol and sulfuric acid in the cold. This substance then is used to prepare Ethyl Nitrite Spirit (Sweet Spirit of Nitre), a product no longer official and which has been removed from the market.

Distillation—Brandy and Whisky are made by distillation. The latter is derived from the fermented mash of wholly or partially germinated malted cereal grains and the former from the fermented juice of ripe grapes.

Incompatibilities—Spirits are, for the most part, preparations of high alcoholic strength and do not lend themselves well to dilution with aqueous solutions or liquids of low alcoholic content. The addition of such a solution invariably causes separation of some of the material dissolved in the spirit, evidenced by a turbidity which, in time, may disappear as distinct layering occurs. Salts may be precipitated from their aqueous solutions by the addition of spirits due to their lesser solubility in alcoholic liquids.

Some spirits show incompatibilities characteristic of the ingredients they contain. For example, Aromatic Ammonia Spirit cannot be mixed with aqueous preparations containing alkaloids (eg, codeine phosphate). An acid-base reaction (ammonia-phosphate) occurs and, if the alcohol content of the final mixture is too low, codeine will precipitate.

Toothache Drops

Toothache drops are preparations used for temporary relief of toothache by application of a small pledget of cotton saturated with the product into the tooth cavity. Anesthetic compounds include clove oil, eugenol or benzocaine; other ingredients include camphor, creosote, menthol and alcohol. Clove oil, containing a high concentration of eugenol, which is the main constituent, has been considered safe and effective for toothache.

These preparations no longer are recognized officially. Furthermore, dentists do not recommend the use of toothache drops if the patient has ready access to adequate dental services. Some preparations may damage the gums and produce complications more severe than the original toothache. However, many areas do not have adequate dental services and the pharmacist will, of necessity, handle these preparations, and should warn the patient of possible hazards associated with their use.

Toothache Drops NF XI contains 25 g of chlorobutanol in sufficient clove oil to make the product measure 100 mL. Another formulation contains creosote, clove oil, benzocaine and alcohol in a flexible collodion base.

Emulsions

An emulsion is a two-phase system prepared by combining two immiscible liquids, one of which is dispersed uniformly throughout the other and consists of globules that have diameters equal to or greater than those of the largest colloidal particles. The liquid that is dispersed into small droplets is

called the dispersed, internal or discontinuous phase. The other liquid is the dispersion medium, external phase or continuous phase.

Most emulsions incorporate an aqueous phase into a non-aqueous phase (or *vice versa*). However, it is possible to

prepare emulsions that are basically nonaqueous. For example, investigations of the emulsifying effects of anionic and cationic surfactants on the nonaqueous immiscible system, glycerin and olive oil, have shown that certain amines and three cationic agents produced stable emulsions. This broadening of the basic definition for the term *emulsion* is recognized in the USP.

While the USP definition, given below, is broad enough to encompass nonaqueous systems, emphasis is placed on those emulsions which contain water, as they are by far the most common in pharmacy.

The USP defines emulsions as follows:

Emulsions are two-phase systems in which one liquid is dispersed throughout another liquid in the form of small droplets. Where oil is the dispersed phase and an aqueous solution is the continuous phase, the system is designated as an oil-in-water (O/W) emulsion. Conversely, where water or an aqueous solution is the dispersed phase and oil or oleaginous material is the continuous phase, the system is designated as a water-in-oil (W/O) emulsion.

Applications

When it is necessary to administer oils by the oral route, patient acceptance is enhanced when the oil is prepared in emulsion form. Thus, mineral oil, a laxative, valproic acid an anticonvulsant, oil-soluble vitamins, vegetable oils and preparations for enteral feeding are formulated frequently in an O/W emulsion form to enhance their palatability.

The bioavailability of oils for absorption may be enhanced when the oil is in the form of small droplets. Furthermore, the absorption of some drugs, eg, griseofulvin, sulfonamides and vitamin A, may be enhanced when they are prepared in the form of an O/W emulsion. Emulsion formulations of drugs such as erythromycin and physostigmine salicylate have been considered, in order to improve their stability. Finally, the greatest use of emulsions is for topical preparations. Both O/W and W/O emulsions are used widely, depending upon the effect desired. Emulsion bases of the W/O type tend to be more occlusive and emollient than O/W emulsion bases, which tend to be removed more easily by water. Further information may be found in Chapter 90. The effects of viscosity, surface tension, solubility, particle size, complexation and excipients on the bioavailability of oral suspensions and emulsions have been discussed in detail by Rettig.¹⁷

Practically, emulsions possess a number of important advantages over other liquid forms. These may be summarized in the following way:

1. In an emulsion, the therapeutic properties and the spreading ability of the constituents are increased.
2. The unpleasant taste or odor of an oil can be masked partially or wholly, by emulsification. Secondary masking techniques are available to the formulator but these must be used with caution. If flavors and sweetening agents are added to the emulsion, only minimal amounts should be used in order to prevent the nausea or gastric distress that results on ingestion of larger quantities of these.
3. The absorption and penetration of medicaments are controlled more easily if they are incorporated into an emulsion.
4. Emulsion action is prolonged and the emollient effect is greater than that observed with comparable preparations.
5. Water is an inexpensive diluent and a good solvent for the many drugs and flavors that are incorporated into an emulsion.

While this section on emulsions focuses primarily on those for oral use and to a lesser degree those for topical application, it should be noted that there are a number of emulsions used parenterally which are described in specialized textbooks on this topic. For example, emulsions of the O/W type are used for intravenous feeding of lipid nutrients. These are used to provide a source of calories and essential fatty acids. These emulsions must meet exacting standards in regard to particle size, safety and stability. Examples of commercial products include Intralipid (Cutter) and Liposyn (Abbott). Other specialized uses of emulsions include radiopaque emulsions which are used as diagnostic agents for

X-ray examination. Other types of emulsions employed parenterally include W/O emulsions of allergenic extracts which are given subcutaneously and radiopaque O/W sustained-release depot preparations given intramuscularly.

Ingredients

The selection of the oil phase for oral preparations depends upon the purpose of the product. For example, mineral oil is used as a laxative and corn oil is used for its nutrient properties. Vegetable oils can be used to dissolve or suspend pharmaceuticals such as oil-soluble vitamins. The selection of the oil phase for topical O/W or W/O preparations is discussed in Chapter 90.

Emulsions are thermodynamically unstable because of the large increase in surface energy due to the combination of interfacial tension and large surface area of the dispersed phase and the different densities of the two phases. As a result, emulsions tend to cream, ie, the less dense phase rises and the more dense phase falls in the container. Subsequently, the droplets can coalesce with a considerable reduction in surface free energy. Consequently, considerable research has been conducted on their preparation and stabilization. The theory of emulsification is described in Chapter 20. In order to prepare suitable emulsions and to have them remain stable for a suitable period of time, a number of excipients are used in their preparation. The most important are those called emulsifying agents, which may be divided into three classes.

1. **Natural Emulsifying Agents**—These substances may be derived from vegetable sources and include acacia, tragacanth, alginates, chondrus and pectin. While the surface activity of these is low, they achieve their emulsifying power by increasing the viscosity of the aqueous phase, as indicated by White.¹⁸ Examples of emulsifying agents derived from animal sources include gelatin, egg yolk, casein, wool fat, cholesterol and lecithin. Because of the widely different chemical constitution of these compounds, they have a variety of uses, depending upon the specific compound, in both oral and topical preparations. All naturally occurring agents show variations in their emulsifying properties from batch to batch.

2. **Finely Divided Solids**—The compounds most frequently used in pharmacy are the colloidal clays: bentonite (aluminum silicate) and veegum (magnesium aluminum silicate). These compounds are good emulsifiers and tend to be absorbed at the interface, effect an increase in viscosity, generally in the aqueous phase, and usually are used in conjunction with a surfactant to prepare O/W emulsions, but both O/W and W/O preparations can be prepared by adding the clay to the external phase first. They are used frequently for external purposes such as a lotion or cream.

3. **Synthetic Emulsifying Agents**—This group of emulsifying agents is most effective at lowering the interfacial tension between the oil and water phases because the molecule possess both hydrophilic and hydrophobic properties. This property is described by their hydrophilic-lipophilic balance (HLB) number which may vary from 40 for sodium dodecyl sulfate to 1 for oleic acid. Emulsifying agents, sometimes used singly, are preferably a combination of two emulsifying agents, which will give a weighted HLB of 8 to 16 which is satisfactory for O/W emulsions and an HLB 3 to 8 for W/O emulsions. These emulsifying agents are available in different ionic types: anionic, eg, sodium dodecyl sulfate; cationic, eg, benzalkonium chloride; nonionic, eg, polyethylene glycol 400 monostearate and ampholytic, eg, long-chain amino acid derivatives. Many of these agents are described in Chapter 80 and the mechanism of action is discussed in Chapter 20.

In addition to the emulsifying agents, viscosity agents are employed, namely the hydrophilic colloids such as naturally occurring gums, noted above, and partially synthetic polymers such as cellulose derivatives, eg, methylcellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose or a number of synthetic polymers that may be used, such as carbomer polymers. These materials are hydrophilic in nature and dissolve or disperse in water to give a viscous solution and function as emulsion stabilizers.

The aqueous phase of the emulsion favors the growth of microorganisms and, because of this, a preservative usually is added to the product. Some of the preservatives that have been used include chlorocresol, chlorobutanol, mercurial preparations, salicylic acid, the esters of *p*-hydroxybenzoic acid, benzoic acid, sodium benzoate or sorbic acid. The preservative should be selected with regard to the ultimate use of the preparation and possible incompatibilities between

the
ing
Low
cha
em
M
pha
pres
the
gan
tive
bou
of i
p-hy
pres
havi
in e
fact
fina
forr
W
with
lipid
pair
tion
use
bec
T
pha
only
and
the
and
dan
on s
of t
tast
info
text
O
incl

Pre
Af
topic
ingr
scri
med

1.
ous:
2.
mau
3.
later
of er
a sai
cally
sele
used
prod
froze
Mec
the
emu
ity o
4.
Heat
high
5.
suffi
6.
thai
7.
8.
solu
9.
the

the preservative and the ingredients in the emulsion, eg, binding between the surface-active agent and the preservative. Low pH values of 5 to 6 and low concentrations of water are characteristics also likely to inhibit microbiological growth in emulsions.

Most emulsions consist of a nonaqueous (or oil or lipid) phase and an aqueous (or water) phase, thus some of the preservative may pass into the oil phase and be removed from the aqueous phase. It is in the aqueous phase that microorganisms tend to grow. As a result, water-soluble preservatives are more effective since the concentration of the unbound preservative in the aqueous phase assumes a great deal of importance in inhibiting the microbial growth. Esters of *p*-hydroxybenzoic acid appear to be the most satisfactory preservatives for emulsions. Many mathematical models have been used to determine the availability of preservatives in emulsified systems. However, because of the number of factors which reduce the effectiveness of the preservative, a final microbiological evaluation of the emulsion must be performed.

While emphasis concerning preservation of emulsions deals with the aqueous phase, microorganisms can reside also in the lipid phase. Consequently, it has been recommended that pairs of preservatives be used to ensure adequate concentration in both phases. Esters of *p*-hydroxybenzoic acid can be used to ensure appropriate concentrations in both phases because of their difference in oil and water solubilities.

The oxidative decomposition of certain excipients in the oil phase in some pharmaceuticals is possible in emulsions, not only because of the usual amount of air dissolved in the liquid, and the possible incorporation of air during the preparation of the product, but also the large interfacial area between the oil and water phase. The selection of the appropriate antioxidant briefly described at the beginning of the chapter depends on such factors as stability, compatibility with the ingredients of the emulsion, toxicity, effectiveness in emulsions, odor, taste and distribution between the two phases. Additional information can be found in appropriate references and in textbooks listed in the *Bibliography*.

Other excipients for the proper formation of emulsions include flavoring agents and fragrances.

Preparation

After the purpose of the emulsions has been determined, ie., oral or topical use, and the type of emulsions, O/W or W/O, and appropriate ingredients selected and the theory of emulsification considered, described in Chapter 21, experimental formulations may be prepared. One method is suggested by Griffin.¹⁹

1. Group the ingredients on the basis of their solubilities in the aqueous and nonaqueous phases.
2. Determine the type of emulsion required and calculate an approximate HLB (hydrophile-lipophile balance) value.
3. Blend a low HLB emulsifier and a high HLB emulsifier to the calculated value. For experimental formulations, use a higher concentration of emulsifier (eg, 10 to 30% of the oil phase) than that required to produce a satisfactory product. Emulsifiers should, in general, be stable chemically, nontoxic and suitably low in color, odor and taste. The emulsifier is selected on the basis of these characteristics, the type of equipment being used to blend the ingredients and the stability characteristics of the final product. Emulsions should not coalesce at room temperature, when frozen and thawed repeatedly or at elevated temperatures of up to 50°. Mechanical energy input varies with the type of equipment used to prepare the emulsion. The more the energy input, the less the demand on the emulsifier. Both process and formulation variables can affect the stability of an emulsion.
4. Dissolve the oil-soluble ingredients and the emulsifiers in the oil. Heat, if necessary, to approximately 5 to 10° over the melting point of the highest melting ingredient or to a maximum temperature of 70 to 80°.
5. Dissolve the water-soluble ingredients (except acids and salts) in a sufficient quantity of water.
6. Heat the aqueous phase to a temperature which is 3 to 5° higher than that of the oil phase.
7. Add the aqueous phase to the oily phase with suitable agitation.
8. If acids or salts are employed, dissolve them in water and add the solution to the cold emulsion.
9. Examine the emulsion and make adjustments in the formulation if the product is unstable. It may be necessary to add more emulsifier, to

change to an emulsifier with a slightly higher or lower HLB value or to use an emulsifier with different chemical characteristics.

The technique of emulsification of pharmaceutical preparations has been described by White.¹⁸ The preparation of an emulsion requires work to reduce the internal phase into small droplets and disperse them throughout the external phase. This can be accomplished by a mortar and pestle or a high-speed emulsifier. The addition of emulsifying agents not only reduces this work but also stabilizes the final emulsion. Emulsions may be prepared by four principle methods.

Addition of Internal Phase to External Phase—This is usually the most satisfactory method for preparing emulsions since there is always an excess of the external phase present which promotes the type of emulsion desired. If the external phase is water and the internal phase is oil, the water-soluble substances are dissolved in the water and the oil-soluble substances mixed thoroughly in the oil. The oil mixture is added in portions to the aqueous preparation with agitation. Sometimes, in order to give a better shearing action during the preparation, all of the water is not mixed with the emulsifying agent until the primary emulsion with the oil is formed; subsequently, the remainder of the water is added. An example using gelatin Type A is given below.

Addition of the External Phase to the Internal Phase—Using an O/W emulsion as an example, the addition of the water (external phase) to the oil (internal phase) will promote the formation of a W/O emulsion due to the preponderance of the oil phase. After further addition of the water, phase inversion to an O/W emulsion should take place. This method especially is useful and successful when hydrophilic agents such as acacia, tragacanth or methylcellulose are first mixed with the oil, effecting dispersion without wetting. Water is added and, eventually, an O/W emulsion is formed. This "dry gum" technique is a rapid method for preparing small quantities of emulsion. The ratio 4 parts of oil, 2 parts of water and 1 part of gum provides maximum shearing action on the oil globules in the mortar. The emulsion then can be diluted and triturated with water to the appropriate concentrations. The preparation of Mineral Oil Emulsion described below is an example.

Mixing Both Phases after Warming Each—This method is used when waxes or other substances which require melting are used. The oil-soluble emulsifying agents, oils and waxes are melted and mixed thoroughly. The water-soluble ingredients dissolved in the water are warmed to a temperature slightly higher than the oil phase. The two phases then are mixed and stirred until cold. For convenience, but not necessity, the aqueous solution is added to the oil mixture. This method frequently is used in the preparation of ointments and creams. An example of an oral preparation containing an insoluble drug is given below.

Alternate Addition of the Two Phases to the Emulsifying Agent—A portion of the oil, if an O/W emulsion is being prepared, is added to all of the oil-soluble emulsifying agents with mixing, then an equal quantity of water containing all the water-soluble emulsifying agents is added with stirring until the emulsion is formed. Further portions of the oil and water are added alternately until the final product is formed. The high concentration of the emulsifying agent in the original emulsion makes the initial emulsification more likely and the high viscosity provides effective shearing action leading to small droplets in the emulsion. This method often is used successfully with soaps.

Examples of some emulsions are given below.

In NF XIII it was suggested that only O/W emulsions are suitable for oral use because these are water-miscible and thus their oiliness is masked. This compendium gave specific directions for the preparation of emulsions using gelatin as an emulsifying agent. These preparations are based on either Type A or Type B gelatin.

Type A gelatin is prepared by acid-treated precursors and is used at a pH of about 3.2. It is incompatible with anionic emulsifying agents such as the vegetable gums. The following formula was recommended.

Gelatin (Type A)	8 g
Tartaric Acid	0.6 g
Flavor as desired	
Alcohol	60 mL
Oil	500 mL
Purified Water, to make	1000 mL

Add the gelatin and the tartaric acid to about 300 mL of purified water, allow to stand for a few minutes, heat until the gelatin is dissolved, then raise the temperature to about 98° and maintain this temperature for about 20 min. Cool to 50°, add the flavor, the alcohol and sufficient purified water to make 500 mL. Add the oil, agitate the mixture thoroughly and

pass it through a homogenizer or a colloid mill until the oil is dispersed completely and uniformly.

This emulsion cannot be prepared by trituration or by the use of the usual stirring devices.

Type B gelatin is prepared from alkali-treated precursors and is used at a pH of about 8.0. It may be used with other anionic emulsifying agents but is incompatible with cationic types. If the emulsion contains 50% oil, 5 g of Type B gelatin, 2.5 g of sodium bicarbonate and sufficient tragacanth or agar should be incorporated into the aqueous phase to yield 1000 mL of product of the required viscosity.

An emulsion that may be prepared by the mortar and pestle method is the following Mineral Oil Emulsion USP.

Mineral Oil	500 mL
Acacia, in very fine powder	125 g
Syrup	100 mL
Vanillin	40 mg
Alcohol	60 mL
Purified Water, a sufficient quantity	1000 mL

The mineral oil and acacia are mixed in a dry Wedgwood mortar. Purified water (250 mL) is added and the mixture triturated vigorously until an emulsion is formed. A mixture of the syrup, 50 mL of purified water and the vanillin dissolved in alcohol is added in divided portions with trituration; sufficient purified water is then added to the proper volume, the mixture mixed well and homogenized.

An Oral Emulsion (O/W) Containing an Insoluble Drug²⁰

Cottonseed Oil	460.0 g
Sulfadiazine	200.0 g
Sorbitan Monostearate	84.0 g
Polyoxyethylene 20 Sorbitan Monostearate	36.0 g
Sodium Benzoate	2.0 g
Sweetener	qs
Purified Water	1000.0 g
Flavor Oil	qs

The procedure as indicated by Rieger²⁰ is

1. Heat the first three ingredients to 50° and pass through colloid mill.
2. Add the next four ingredients at 50° to the first three ingredients at 65° and stir while cooling to 45°.
3. Add the flavor oil and continue to stir until room temperature is reached.

Properties

The type of emulsion O/W or W/O depends, to some extent, on the phase-volume ratio. The higher the fraction of one phase, the greater likelihood it will form the external phase. Thus, O/W emulsions are favored if water forms a greater fraction of the volume than the oil phase. However, it is possible for the internal phase of an emulsion to occupy up to 0.74 of the volume of the emulsion and still form a stable product. Emulsifiers with high HLB level values (8 to 16) tend to form an O/W emulsion, while those with low HLB values (3 to 8) tend to form a W/O emulsion.

The consistency of emulsions, as suggested by White,¹⁸ can be increased by increasing the viscosity of the continuous phase, increasing the fractional volume of the internal phase, reducing the particle size of the internal phase, increasing the proportion of the emulsifying agent or adding hydrophobic emulsifying agents to the oil phase of the emulsion.

The physical stability of emulsions may be defined by a number of expressions. The first of these, which is called *creaming*, is the movement of the droplets either upward or downward, depending upon their density. This gives a product which is not homogenous and can lead to a nonuniform dose. Generally, creaming is not a serious problem because a moderate amount of shaking will redisperse the droplets uniformly. The rate of creaming may be decreased by considering the theory of creaming using Stokes law, Chapter 20. This equation relates the rate of creaming to the size of the droplets, the difference in densities and the viscosity of the external phase. Thus, the rate of creaming may be decreased

by decreasing the size of the droplets and increasing the viscosity of the external phases, both of which have been discussed above. Minimizing the difference between densities is more difficult to achieve due to a number of practical difficulties.

When the droplets aggregate, they come together and act as a single unit, but do not fuse. As a result of the larger size, they tend to cream faster and further provoke physical instability. Aggregation is to some extent reversible and may be controlled by choosing a somewhat different surfactant system and controlling the electrical potential of the droplets. Coalescence of an emulsion is the fusion of the droplets, leading to a decrease in their numbers and eventually the complete separation of the two phases, yielding an unsatisfactory product which should be reformulated completely (see Chapter 21).

Multiple Emulsions—A recent innovation in emulsion technology is the development of multiple emulsions. The dispersed phase of these emulsions contains even smaller droplets which are miscible with the continuous phase. Thus, the multiple emulsion may be O/W/O where the aqueous phase is between two oil phases, or W/O/W where the internal and external aqueous phases are separated by an oil phase. In these systems both hydrophobic and hydrophilic emulsifiers are used and both have an effect on the yield and stability, as noted by Florence and Whitehill.²¹

It appears that O/W/O emulsions are formed better by lipophilic, nonionic surfactants using gum acacia-emulsified simple systems, while W/O/W multiple emulsions are formed better by nonionic surfactants in a two-stage emulsification procedure. A specific formulation for a W/O/W emulsion may be prepared by forming the primary (W/O) emulsion from isopropyl myristate (47.5%), sorbitan monooleate (2.5%) and distilled water to 100%. This primary emulsion (50%) is added to a polyoxyethylene sorbitan monooleate (2% w/v) solution in water as suggested by Florence and Whitehill.²¹ Other formulations of multiple emulsions include carboxymethylcellulose sodium, microcrystalline cellulose, sorbitan monooleate and sorbitan trioleate.

While the technique of preparing these emulsions is more complicated, research indicates potential use of these emulsions for prolonged action, taste-masking, more effective dosage forms, improved stability, parenteral preparations, protection against the external environment and enzyme entrapment. These emulsions also may be used to separate two incompatible hydrophilic substances in the inner and outer aqueous phases by the middle oil phase.

Microemulsions—The coarse pharmaceutical macro emulsions appear white and tend to separate on standing. Microemulsions are translucent or transparent, do not separate and have a droplet diameter in the nanometer size range. The microemulsions are not always distinguishable from micellar solutions.

Both O/W and W/O types are possible and may be converted, one to the other, by adding more of the internal phase or by altering the type of emulsifier. As the internal phase is added, the emulsion will pass through a viscoelastic gel stage; with further addition, an emulsion of the opposite type will occur.

The most obvious benefit of microemulsions is their stability, thus providing dose uniformity. Usually, the emulsifier should be 20 to 30% of the weight of the oil used. The W/O systems are prepared by blending the oil and emulsifier with a little heat, if required, and then adding the water. The order of mixing for O/W systems is more flexible. One of the simplest methods is to blend the oil and the emulsifier and pour this into water with a little stirring. In no case can a microemulsion be formed unless there is a match between the oil and emulsifier.

If the emulsifier has been selected properly, microemulsification will occur almost spontaneously, leading to a satisfactory and stable preparation. The details of various preparations and the relationship between microemulsions and

mic
emu
On
sion
coar
prim
the
tant
drop
mole
has
of m
G
emu
fuga
men
cal
opti

Eq

TL
ener
addi
the
the
the
alar
amo
atio
ener
tem
proc
emu
phas
Bu
volu
uct,
emu
disc
the
Si
proc
tion
equi
has
men
effe
Ti
ties
exp
tech
ticle
mer
ingr
orde
of k
par
A
pre
by t
dis
mit
con
not
mer
Lab
duc
M
ing
mix
eler
for

micellar solutions have been reviewed by Prince *et al.*²² Micro-emulsions containing hydrocortisone have been prepared.

Other authors suggest that the preparation of microemulsions is considerably more difficult than the preparation of coarse suspensions. Rosano *et al.*²³ discusses the use of a primary surfactant adsorbed at the interface which influences the curvature of the dispersed phase. The amount of surfactant required may be estimated from the surface area of the droplets and the cross-sectional area of the surfactant molecule. The use of a cosurfactant to form a duplex film has been indicated. The authors also suggest that the order of mixing is important.

General methods are available for testing the instability of emulsions including bulk changes, centrifugal and ultracentrifugal studies, dielectric measurement, surface-area measurement and accelerated-motion studies. Low-shear rheological studies measuring viscoelasticity are suggested as the optimal method of stability testing.

Equipment

The preparation of emulsions requires a certain amount of energy to form the interface between the two phases, and additional work must be done to stir the system to overcome the resistance to flow. In addition, heat often is supplied to the system to melt waxy solids and/or reduce the viscosity of the oil phase. Consequently the preparation of emulsions on a large scale usually requires the expenditure of considerable amounts of energy for heating and mixing. Careful consideration of these processes has led to the development of low-energy emulsification by using an appropriate emulsification temperature and selective heating of the ingredients. This process, described by Lin,²⁴ involves the preparation of an emulsion concentrate subsequently diluted with the external phase at room temperature.

Because of the variety of oils used, emulsifier agents, phase-volume ratios and the desired physical properties of the product, a wide selection of equipment is available for preparing emulsions and an outline of the main classes of equipment is discussed below. Further information may be obtained from the *Bibliography*.

Special techniques and equipment in certain instances, will produce superior emulsions, including rapid cooling, reduction in particle size or ultrasonic devices. A wide selection of equipment for processing both emulsions and suspensions has been described by Eisberg.²⁵ A number of improvements have been made to make the various processes more effective and energy-efficient.

The mortar and pestle may be used to prepare small quantities of an emulsion, and it is one of the simplest and least expensive methods. It may be used for most of the different techniques of preparing emulsions. Generally, the final particle size is considerably larger than is achieved by the equipment described below. In addition, it is necessary for the ingredients to have a certain viscosity prior to trituration in order to achieve a satisfactory shear. Satisfactory emulsions of low-viscosity ingredients and small volumes may be prepared using the appropriate equipment described below.

Agitators—Ordinary agitation or shaking may be used to prepare the emulsion. This method frequently is employed by the pharmacist, particularly in the emulsification of easily dispersed, low-viscosity oils. Under certain conditions, intermittent shaking is considerably more effective than ordinary continuous shaking. Continuous shaking tends to break up not only the phase to be dispersed but also the dispersion medium and, in this way, impairs the ease of emulsification. Laboratory shaking devices may be used for small-scale production.

Mechanical Mixers—Emulsions may be prepared by using one of several mixers which are available. Propeller-type mixers which have a propeller attached to a shaft driven by an electric motor are convenient and portable and can be used for both stirring and emulsification. This type operates best

in mixtures which have low viscosity, ie, mixtures with a viscosity of glycerin or less. They are also useful for preparing emulsions. A turbine mixer has a number of blades which may be straight or curved, with or without a pitch, mounted on a shaft. The turbine tends to give a greater shear than propellers. The shear can be increased by using diffuser rings which are perforated and surround the turbine so that the liquid from the turbine must pass through holes. The turbines can be used for both low-viscosity mixtures and medium-viscosity liquids, up to that of molasses. The degree of stirring and shear by propeller or turbine mixers depends upon several factors, such as the speed of rotation, pattern of liquid flow, position in the container and baffles in the container as discussed by Fox.²⁶

Production-size mixers include high-powered propeller-shaft stirrers immersed in a tank or self-contained units with propeller and paddle systems. The latter usually are constructed so that the contents of the tank either may be heated or cooled during the production process. Baffles often are built into a tank and these increase the efficiency of mixing. Two mixers manufactured by the same company are shown in Figs 2 and 3.

Small electric mixers may be used to prepare emulsions at the prescription counter. They will save time and energy and produce satisfactory emulsions when the emulsifying agent is acacia or agar.

The commercially available *Waring Blendor* disperses efficiently by means of the shearing action of rapidly rotating blades. It transfers large amounts of energy and incorporates air into the emulsion. If an emulsion first is produced by using a blender of this type, the formulator must remember that the emulsion characteristics obtained in the laboratory will not be duplicated necessarily by the production-size equipment.

Colloid Mills—The principle of operation of the colloid mill is the passage of the mixed phases of an emulsion formula between a stator and a high-speed rotor revolving at speeds of 2000 to 18,000 rpm. The clearance between the rotor and the stator is adjustable, usually from 0.001 in upward. The emulsion mixture, in passing between the rotor and stator, is subjected to a tremendous shearing action which effects a fine dispersion of uniform size as indicated by Griffin *et al.*¹⁹ A colloid mill and various rotors are shown in Figs 4 and 5. The operating principle is the same for all, but each manufacturer incorporates specific features which result in changes in oper-

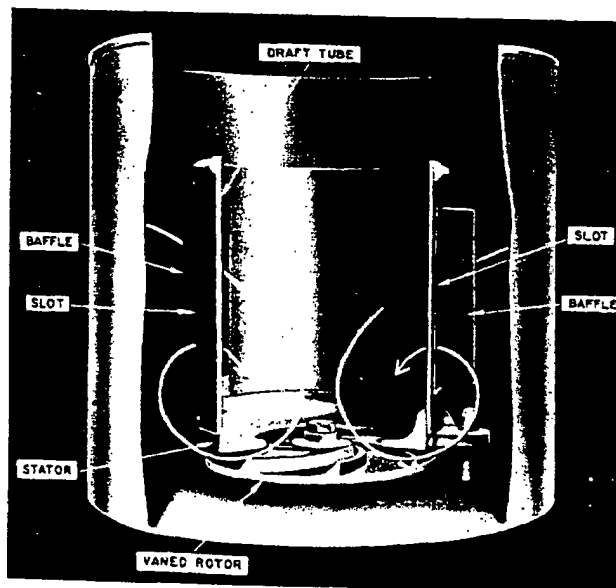


Fig 2. Standard slurry-type dispersall mixer with vaned-rotor "mixing" element and slotted draft-tube circulating element (courtesy, Abbe Eng).

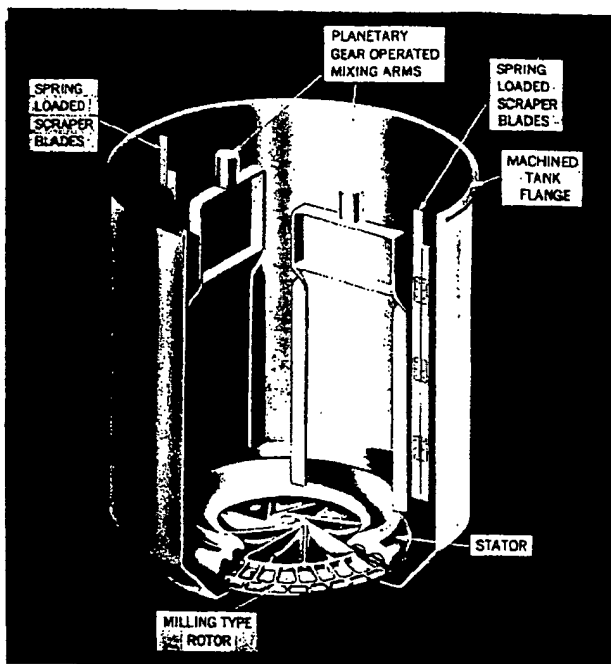


Fig 3. Standard paste-type dispersal mixer with "cupped-rotor" milling element and double-rotating mixing arm circulating element (courtesy, Abbe Eng).

ating efficiency. The shearing forces applied in the colloid mill usually result in a temperature increase within the emulsion. It may be necessary, therefore, to cool the equipment when the emulsion is being produced.

Colloid mills are used frequently for the comminution of solids and for the preparation of suspensions, especially suspensions containing solids which are not wetted by the dispersion medium.

Homogenizers—Impeller types of equipment frequently produce a satisfactory emulsion; however, for further reduction in particle size, homogenizers may be employed, as indicated by Scott.²⁷

Homogenizers may be used in one of two ways:

1. The ingredients in the emulsion are mixed and then passed through the homogenizer to produce the final product.

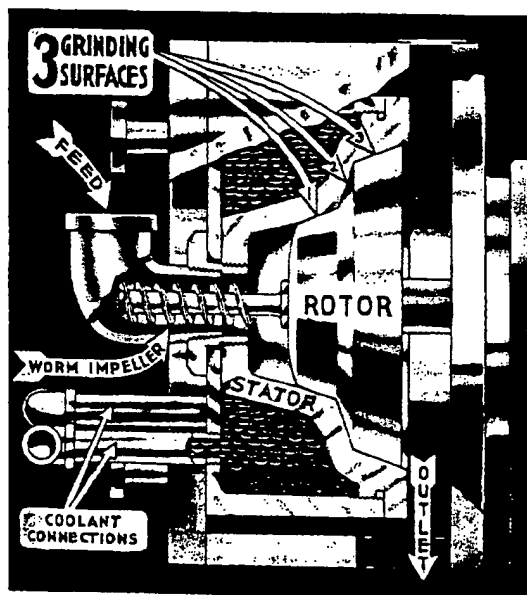


Fig 4. A colloid mill shown in cross section (courtesy, Tri-Homo).

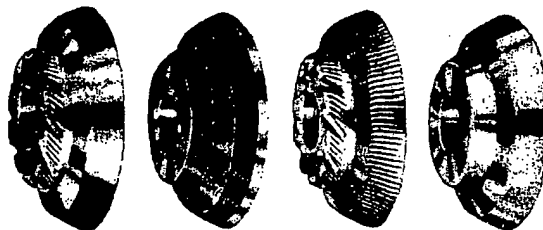


Fig 5. Types of rotors used in colloid mills. These may be smooth (for most emulsions), serrated (for ointments and very viscous products) or of vitrified stone (for the paints and pigment dispersions) (courtesy, Tri-Homo).

2. A coarse emulsion is prepared in some other way and then passed through a homogenizer for the purpose of decreasing the particle size and obtaining a greater degree of uniformity and stability.

The mixed phases or the coarse emulsion are subjected to homogenization and are passed between a finely ground valve and seat under high pressure. This, in effect, produces an atomization which is enhanced by the impact received by the atomized mixture as it strikes the surrounding metal surfaces. They operate at pressures of 1000 to 5000 psi and produce some of the finest dispersions obtainable in an emulsion.

Figure 6 shows the flow through the homogenizing valve, the heart of the high-pressure APV Gaulin homogenizer. The product enters the valve seat at high pressure, flows through the region between the valve and the seat at high velocity with a rapid pressure drop, causing cavitation; subsequently, the mixture hits the impact ring causing further disruption and then is discharged as a homogenized product. It is postulated that circulation and turbulence are responsible mainly for the homogenization that takes place. Different valve assemblies, two stage valve assemblies and equipment with a wide range of capacities are available.

Two-stage homogenizers are constructed so that the emulsion, after treatment in the first valve system, is conducted directly to another where it receives a second treatment. A single homogenization may produce an emulsion which, although its particle size is small, has a tendency to clump or form clusters. Emulsions of this type exhibit increased creaming tendencies. This is corrected by passing the emulsion through the first stage of homogenization at a high pressure (eg, 3000 to 5000 psi) and then through the second stage at a greatly reduced pressure (eg, 1000 psi). This breaks down any clusters formed in the first step.

The Macro Flow-Master *Kom-bi-nator* employs a number of different actions, each of which takes the ingredients a little further along in the process of subdividing droplets, until complete homogenization results. The machine is equipped with a pump which carries the liquid through the various stages of the process. In the first stage, the ingredients are forced between two specially designed rotors (gears) which shoot the liquid in opposite directions in a small chamber and, in this way, are mixed thoroughly. These rotors also set up a swirling action in the next chamber into which the liquid is forced and swirled back and forth in eddies and crosscurrents. The second stage is a pulsing or vibrating action at rapid frequency. The product then leaves this chamber, goes through a small valve opening and is dashed against the wall of

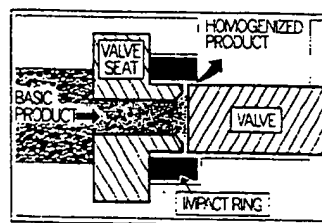


Fig 6. Operation of the homogenizer valve assembly (Courtesy APV Gaulin).

the homogenizing chamber. Pressure is applied, but it is not as great as that used in other types of homogenizers. Pressure is controlled accurately by adjusting devices on the front of the machine, and temperature is controlled by passing coolants through the stators.

For small-scale extemporaneous preparation of emulsions, the inexpensive *hand-operated homogenizer* (available from *Central Scientific*) is particularly useful. It is probably the most efficient emulsifying apparatus available to the prescription pharmacist. The two phases, previously mixed in a bottle, are hand pumped through the apparatus. Recirculation of the emulsion through the apparatus will improve its quality.

A homogenizer does not incorporate air into the final product. Air may ruin an emulsion because the emulsifying agent is adsorbed preferentially at the air/water interface, followed by an irreversible precipitation termed *denaturation*. This is particularly prone to occur with protein emulsifying agents.

Homogenization may spoil an emulsion if the concentration of the emulsifying agent in the formulation is less than that required to take care of the increase in surface area produced by the process.

The temperature rise during homogenization is not very large. However, temperature does play an important role in the emulsification process. An increase in temperature will reduce the viscosity and, in certain instances, the interfacial tension between the oil and the water. There are, however, many instances, particularly in the manufacturing of cosmetic creams and ointments, where the ingredients will fail to emulsify properly if they are processed at too high a temperature.

Emulsions of this type are processed first at an elevated temperature and then homogenized at a temperature not exceeding 40°.

Homogenizers have been used most frequently with liquid emulsions, but now they may be used with suspensions, as the metal surfaces are formed from wear-resistant alloys which will resist the wear of solid particles contained in suspensions.

Ultrasonic Devices—The preparation of emulsions by the use of ultrasonic vibrations also is possible. An oscillator of high frequency (100 to 500 kHz) is connected to two electrodes between which is placed a piezoelectric quartz plate. The quartz plate and electrodes are immersed in an oil bath and, when the oscillator is operating, high-frequency waves flow through the fluid. Emulsification is accomplished by simply immersing a tube containing the emulsion ingredients into this oil bath. Considerable research has been done on ultrasonic emulsification, particularly with regard to the mechanism of emulsion formation by this method. Limited data indicate that these devices will produce stable emulsions only with liquids of low viscosity. The method is not practical, however, for large-scale production of emulsions.

Commercial products may be prepared using ultrasonics based upon the device known as the Pohlman whistle. In this apparatus, the premixed liquids are forced through a thin orifice and are allowed to impinge upon the free end of a knife-edge bar which is made to vibrate. Ultrasonic waves are produced and areas of compression and rarefaction are formed. Shock waves are produced by the collapse of bubbles which produce a shear effect, thereby producing fine particle sizes as described by Scott.²⁷

Suspensions

The physical chemist defines the word "suspension" as a two-phase system consisting of a finely divided solid dispersed in a solid, liquid or gas. The pharmacist accepts this definition and can show that a variety of dosage forms fall within the scope of the preceding statement. There is, however, a reluctance to be all-inclusive, and it is for this reason that the main emphasis is placed on solids dispersed in liquids. In addition, and because there is a need for more specific terminology, the pharmaceutical scientist differentiates between such preparations as suspensions, mixtures, magmas, gels and lotions. In a general sense, each of these preparations represents a suspension, but the state of subdivision of the insoluble solid varies from particles which settle gradually on standing to particles which are colloidal in nature. The lower limit of particle size is approximately 0.1 μm , and it is the preparations containing dispersed solids of this magnitude or greater that are defined pharmaceutically as suspensions.

Suspensions have a number of applications in pharmacy. They are used to supply drugs to the patient in liquid form. Many people have difficulty swallowing solid dosage forms, consequently a liquid preparation has an advantage for these people; in addition, the dose of a liquid form may be adjusted easily to meet the patient's requirements. Thus, if the drug is insoluble or poorly soluble, a suspension may be the most suitable dosage form. If a drug is unstable in an aqueous medium, a different form of the drug, such as an ester or insoluble salt, which does not dissolve in water, may be used in the preparation of a suspension.

In order to improve the stability of an antibiotic such as ampicillin, formulations are made in such a way that the dispersion medium, water, is added upon dispensing to form a satisfactory suspension. Generally, the taste of pharmaceuticals can be improved if they are supplied in suspension form, rather than solutions; thus, chloramphenicol palmitate is used instead of the more soluble form, chloramphenicol. Another way to decrease the solubility of the drug is to replace part of the water with another appropriate liquid such as alcohol or

glycerin. Insoluble drugs may be formulated as suspensions for topical use such as calamine lotion.

Other preparations of suspensions, in addition to those noted above, include parenteral preparations, ophthalmic preparations or medicated applications discussed in Chapters 87, 89 and 90, respectively.

Certain authors also include liniments, and the newer sustained-release suspensions, in any discussion of this particular subject. The former preparations now usually are considered as solutions although a number of older liniments were, in fact, suspensions. The sustained-release suspensions represent a very specialized class of preparation and, as such, are discussed in more detail in Chapter 94. Some insoluble drugs also are administered in aerosol form; one example is dexamethasone phosphate suspended in a propellant mixture of fluorochlorocarbons. More detail on aerosols is available in Chapter 95.

Suspension formulation and control is based on the principles outlined in Chapters 20 and 22. Formulation involves more than suspending a solid in a liquid. A knowledge of the behavior of particles in liquids, of suspending agents and of flavors and colors is required to produce a satisfactory suspension.

Well-formulated suspensions should possess certain basic properties. The dispersed phase should settle slowly, if at all, and be redispersed readily on shaking. The particles should not cake on settling and the viscosity should be such that the preparation pours easily. As with all dosage forms, there should be no question as to the chemical stability of the suspension.

Ingredients

The main ingredients in a suspension are the drug and agents to wet the drug, influence flocculation, control viscosity, adjust pH, and the external medium, usually water. In addition, flavoring, sweetening and coloring agents and preservatives are employed.

A *wetting agent*, ie, a suitable surfactant with a HLB value between 7 and 9, is used; although surfactants with higher HLB values are recommended sometimes, eg, certain polysorbates and poloxamers. They are employed at a low concentration (0.05 to 0.5%) to allow the displacement of air from hydrophobic material and permit the liquid, usually water, to surround the particles and provide a proper dispersion. If it is desirable to flocculate the particles, then flocculating agents are employed. Usually low concentrations, less than 1%, of electrolytes such as sodium or potassium chloride are employed to induce flocculation. Water-soluble salts possessing divalent or trivalent ions may be considered if the particles are highly charged.

Viscosity agents such as natural gums, eg, acacia, xanthan and cellulose derivatives such as sodium carboxymethylcellulose and hydroxypropylmethylcellulose, may be used at low concentrations (<0.1%) to function as protective colloids, but at higher concentrations they can then function as viscosity-increasing agents and they decrease the rate of settling of deflocculated particles or provide stability in a flocculated suspension.

The choice of an appropriate viscosity agent depends upon the end-use of the product (external or internal), facilities for preparation and the duration of storage.

Extemporaneous preparations of suspensions for internal use showing good flow and suspending properties are provided by sodium carboxymethylcellulose 2.5%, tragacanth 1.25% and guar gum 0.5%. Avicel RC-591, a coprecipitate of microcrystalline cellulose and sodium carboxymethylcellulose stabilized with hydroxypropylmethylcellulose, has been used as a suspending vehicle for propranolol and orphenadrine hydrochloride dispersions prepared from tablets. It also may serve as a general-purpose suspending agent. Carbopol 934, 0.3% or greater, was a satisfactory suspending agent for sulfamethazine 10%, maintaining a permanent suspension for more than 6 months. Other agents include acacia, methylcellulose or other cellulose derivatives and sodium alginate or tragacanth.

Buffers may be considered if the drug has ionizable groups in order to maintain a low solubility of the drug. Buffers also may be considered to control the ionization of preservatives, ionic viscosity agents or to maintain the pH of the suspensions within a suitable range. The external phase is usually water for oral preparations; however, other polar liquids such as glycerin or alcohol may be considered in order to control solubility, stability and taste. The selection of the external phase is based upon taste, viscosity, density and stability. Nonpolar liquids such as aliphatic hydrocarbons and fatty esters may be considered if the preparation is used for external purposes.

Appropriate preservatives should be incorporated in order to minimize microbiological contamination as discussed previously. The suspension must be acceptable to the patient on the basis of its taste, color and cosmetic qualities (elegance), the latter two factors being of particular importance in preparations intended for external use.

Preparation

The preparation of suspensions involves several steps; the first is to obtain the particles in the proper size range which is in the lower micrometer size. Oral preparations should not feel gritty, topical preparations should feel smooth to the touch and injectables should not produce tissue irritation. Particle size and distribution also should be considered in terms of bioavailability, or alternately, to control the rate of release. Particles of an extremely small size, less than 1 μm will have a higher solubility than larger particles, which may cause problems in regard to dissolution and then the formation of larger particles.

Milling is the term given to the mechanical process of reducing the particle size, which may be accomplished by a number of different types of machines, as described by Parrot.²⁸ The hammer mill grinds the powders by the impact

of rotating hammers and particles which subsequently fall through a screen in a range of 4 to 325 mesh are obtained. A ball mill contains a number of steel balls in a container which revolves, and the balls reduce the particle size to a 20 to 200 mesh by both attrition and impact. A fluid-energy mill produces particles 1 to 30 μm through violent turbulence in high-velocity air. Roller mills have two or more rollers which revolve at different speeds and the particles are reduced to a mesh of 20 to 200 by means of compression and a shearing action.

On a small scale, in a pharmacy, the particles should be wetted thoroughly with a small quantity of water-miscible solvent, such as glycerin or alcohol, which reduces the liquid/air interfacial tension. The suspending agent in the aqueous medium then is added. Alternately, the dry suspending agent can be triturated with the drug particles using a small quantity of glycerin or alcohol and then brought up to volume with the diluent water.

On a large scale, the drug particles are treated with a small portion of water which contains the wetting agent and allowed to stand for several hours in order to release entrapped air. At the same time, the suspending agent should be dissolved or dispersed in the main portion of the external phase and allowed to stand until complete hydration takes place. Subsequently, wetted drug particles should be added slowly to the main portion of the dissolved suspending agent. Other excipients such as electrolytes or buffers should be added in a careful manner to prevent variation in particle charge. The preservatives, flavoring agents and coloring agents are added. After all additions have been made, treatment with homogenizers or ultrasonic devices should be used to reduce the size of agglomerated particles, as described by Nash.²⁹

Suspension equipment such as colloid mills or homogenizers normally are used in wet-milling finished suspensions to reduce particle agglomerates and to form a suitable preparation (Figs. 4, 5 and 6).

Quality

The quality of the suspension can be determined in a number of ways, such as photomicroscopy, to determine particle shape, size and flocculation. The Coulter counter can be used to determine the size distribution. Physical stability, ie, the degree of settling or flocculation, may be determined by using cylindrical graduates. Viscosity of the final product and the suspending agent dissolved in the liquid medium may be determined by moisture instruments such as the Brookfield viscometer. Specific-gravity measurements are useful for determining the degree of air entrapped. Of course both microbiological as well as aging tests should be performed to determine the efficiency of the preservative and the appropriateness of the formulation with respect to stability and time.

Suspensions from Tablets

Occasionally, it is necessary to prepare a liquid formulation of a drug in order to meet certain requirements of the patient such as inability to ingest a solid dosage form or to prepare a product for a different route of administration or different strength. The pure drug should be used to prepare the dosage form rather than a tablet or a capsule because there is only one ingredient in the product; thus, no consideration has to be given to the excipients in the tablet or capsule. If it is necessary to prepare a liquid dosage form from tablets or capsules, a suspension is formed if either the drug or one of the excipients in the tablets or capsules is insoluble. The solubility of the drug may be determined from the literature; however, the excipients in the tablets or capsules are usually not known.

Insoluble excipients in these dosage forms may include certain disintegrants, lubricants, glidants, colors, diluents and coatings; consequently, although the drug may be soluble in water, many excipients are not. It is preferable to use the contents of capsules, or tablets which are not coated, or if coated, those tablets with a water-soluble coat. In any case,

the
finel
alco
med

Fi
prep
beer
ride
vehi
niso
ronc
lol t
may
jour
max
max
Frec
and,

If
prep
rati
app
pre
ous

If
in a
susp
in a
pow
or a
watu

See
A
tabl
M.
Pa
Pu
Pr
Si

A
reta

Gi
Si

T
the
mix
atec

S.
azej
they
resj
Fre
whi
A
bee

Ge

P
two
in t
bel

G.
eith
moli
nerv
sys
part
som
and
bec

the contents of the capsules or the tablets should be ground finely with a mortar and pestle and then wetted using a little alcohol or glycerin as mentioned above, or with the dispersion medium using the mortar and pestle.

Finally, it may be desirable to use a hand homogenizer to prepare a more suitable product. Some drugs which have been formulated in this manner include clonidine hydrochloride and simple syrup, cefuroxime axetil in an orange syrup vehicle, famotidine in cherry syrup, terbutaline in syrup, prednisone in a tuffitrufti formulation, metoprolol tartrate or spirinolactone in a tragacanth-suspending vehicle or propranolol hydrochloride in a simple syrup. Many other examples may be found in current hospital and community pharmacy journals such as the *American Journal of Hospital Pharmacy*, *Canadian Journal of Hospital Pharmacy*, *U.S. Pharmacist Drug Development* and *Industrial Pharmacy*. Frequently, stability data and, occasionally, bioavailability and/or taste data are provided.

If the drug is soluble in water, a solution of the drug may be prepared by crushing the tablets in a mortar and pestle, triturating with water, filtering and bringing the solution up to appropriate volume with water or other suitable vehicle if the preparation is for topical application or with a flavored aqueous vehicle if it is for oral use.

If the active ingredient in the tablet or capsule is not stable in an aqueous system, a different method of preparing the suspension is required. The tablet may be crushed and placed in a powder paper and dispensed in the form of individual powders. Each paper contains the active drug in one tablet or an appropriate dose. The powder is placed in a glass of water or suitable liquid, stirred and administered immediately. See Chapter 91 for divided powders.

A general formula to prepare suspensions from crushed tablets is given in Martindale.³⁰

Methylcellulose 20.....	0.75
Parabens.....	0.1
Purified Water.....	60.0
Propylene Glycol.....	2.0
Simple Syrup, to make.....	100.0

An extemporaneous suspension of cimetidine tablets which retained its potency at 40° over 14 days is:

Cimetidine 300-mg tablets.....	24 (7.2 g)
Glycerin.....	10 mL
Simple Syrup, to make.....	120 mL

The tablets are triturated to a fine powder using a mortar, the mixture is levigated with the glycerin, simple syrup added, mixed well, placed in a blender until smooth and then refrigerated as described by Tortorici.³¹

Satisfactory suspensions have been compounded from diazepam tablets and propranolol hydrochloride tablets, and they possess chemical stability for 60 days and 4 months, respectively, at room temperature or under refrigeration. Frequently, since the drug may be soluble, it is the excipients which are being suspended.

A comprehensive checklist of suspension formulations has been reported in the literature by Scheer.³²

Gels

Pharmaceutical terminology is, at best, confusing and no two texts will classify gels, jellies, magnas, milks and mixtures in the same way. The USP's definition for gels is given below.

Gels (sometimes called Jellies) are semisolid systems consisting of either suspensions made up of small inorganic particles or large organic molecules interpenetrated by a liquid. Where the gel mass consists of a network of small discrete particles, the gel is classified as a two-phase system (eg, *Aluminum Hydroxide Gel*). In a two-phase system, if the particle size of the dispersed phase is relatively large, the gel mass is sometimes referred to as a magma (eg, *Bentonite Magma*). Both gels and magnas may be thixotropic, forming semisolids on standing and becoming liquid on agitation. They should be shaken before use to

ensure homogeneity and should be labeled to that effect. (See *Suspensions*.)

Single-phase gels consist of organic macromolecules distributed uniformly throughout a liquid in such a manner that no apparent boundaries exist between the dispersed macromolecules and the liquid. Single-phase gels may be made from synthetic macromolecules (eg, *Carbomer*) or from natural gums (eg, *Tragacanth*). The latter preparations also are called mucilages. Although these gels are commonly aqueous, alcohols and oils may be used as the continuous phase. For example, mineral oil can be combined with a polyethylene resin to form an oleaginous ointment base.

Gels can be used to administer drugs topically or into body cavities (eg, *Phenylephrine Hydrochloride Nasal Jelly*).

The definition in the BP tends to be more restrictive in the sense that the gels are homogenous and are intended to be applied to the skin or certain mucous membranes. Gels may contain auxiliary substances such as antimicrobial preservatives, antioxidants and stabilizers.

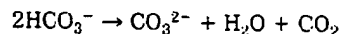
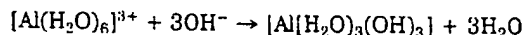
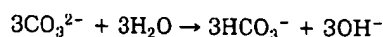
Schott³³ has described various aspects of gels. At appropriate concentrations of solute and solvent, gels consisting of two phases, eg, bentonite, are formed because of the attraction between positively charged edges and the negatively charged faces, producing a three-dimensional network penetrated by the liquid phase. In the case of a single-phase system, the gels are formed as a result of secondary valence forces between the polymer molecules due to entanglement of the chains. Permanent gels are formed when three-dimensional polymerization of multifunctional polymers occurs or when there is cross-linking of dissolved polymer molecules by primary valence bonds. These permanent gels are used as matrices for prolonged-release preparations (see Chapter 94) and are not discussed further in this chapter.

Two-phase gels containing bentonite may be used as a base for topical preparations such as plaster and ointment. Another two-phase gel, Aluminum Hydroxide Gel USP is used for its therapeutic properties.

The USP states that

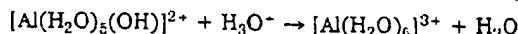
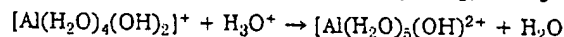
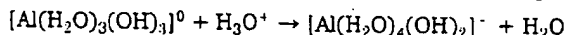
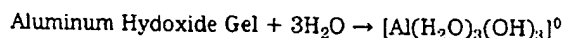
Aluminum Hydroxide Gel is a suspension, each 100 g of which contains the equivalent of not less than 5.5 g and not more than 6.7 g of aluminum hydroxide $[Al(OH)_3]$, in the form of amorphous aluminum hydroxide in which there is a partial substitution of carbonate for hydroxide.

The gel itself usually is prepared by the interaction of a soluble aluminum salt, such as a chloride or sulfate, with ammonia solution, sodium carbonate or bicarbonate. The reactions which occur during the preparation are



The physical and chemical properties of the gel will be affected by the order of addition of reactants, pH of precipitation, temperature of precipitation, concentration of the reactants, the reactants used and the conditions of aging of the precipitated gel.

Aluminum Hydroxide Gel is soluble in acidic (or very strongly basic) media. The mechanism in acidic media is



It is unlikely that the last reaction given proceeds to completion. Since the activity of the gel is controlled by its insolubility (solubility will decrease with an increase in the pH of the gastric media), there is no acid rebound. Further, since a certain quantity of insoluble gel always is available, the neutralizing capability of the gel extends over a considerable period of time.

Aluminum hydroxide gels also may contain peppermint oil, glycerin, sorbitol, sucrose, saccharin and various preservatives. Sorbitol improves the acid-consuming capacity.

ity, apparently by inhibiting a secondary polymerization that takes place on aging. In addition, polyols such as mannitol, sorbitol and inositol have been shown to improve the stability of aluminum hydroxide and aluminum hydroxycarbonate gels.

Other two-phase gels of the USP include Aluminum Phosphate Gel and Aluminum Carbonate gels. Some of these products also occur in the dried form and are also called gels.

Single-Phase Gels—The single-phase gels are being used more frequently in pharmacy and cosmetics because of several properties: semisolid state, high degree of clarity, ease of application and ease of removal and use. The gels often provide a faster release of drug substance, independent of the water solubility of the drug, as compared to creams and ointments.

Some recent gel formulations include ophthalmic preparations of pilocarpine, carbachol and betamethasone valerate; topical preparations for burn therapy, anti-inflammatory treatment, musculoskeletal disorders and acne; peptic ulcer treatment with sucralfate gel and bronchoscopy using lidocaine. Cosmetic gels include shower gels, after shave gels and sunscreen gels. The USP lists a number of gels: Sodium Fluoride and Phosphoric Acid Gel for application to the teeth to reduce cavities, Betamethasone Benzoate Gel and Fluocinonide Gel, anti-inflammatory corticosteroids, Tolnaftate Gel, an antifungal agent and Tretinoin Gel for the treatment of acne. Gels may be used as lubricants for catheters, bases for patch testing, sodium chloride gels for electrocardiography.

Gels can be prepared from a number of pharmaceutical agents such as tragacanth 2 to 5%, sodium alginate 2 to 10%, gelatin 2 to 15%, methylcellulose 450 at 3 to 5%, sodium carboxymethylcellulose 2 to 5%, carbomer 0.3 to 5% or polyvinyl alcohols 10 to 20% as noted by Collett.³⁴ Other gelling agents include methylhydroxyethyl cellulose, polyoxyethylene-polyoxypropylene, hydroxyethylcellulose and gelatin. Gels prepared from nonpolar materials such as magnesium soap-hydrocarbon and hydrocarbons are being investigated. The percentages above indicate the concentration ranges of the gelling agent.

Some fluid gels at or below the lower of the above concentrations can be used as artificial saliva and artificial tears. The lower-percentage preparations, noted above, may be used as lubricants and the higher-percentage preparations as dermatological bases. Some of the gelling agents are available in different grades indicating the viscosity at a definite concentration. In general, high-viscosity grades result in gels at lower concentrations. An example of a gel containing a natural polymer, tragacanth, is:

Ephedrine Sulfate Jelly NF XII

Ephedrine Sulfate	10 g
Tragacanth	10 g
Methyl Salicylate	0.1 g
Eucalyptol	1.0 mL
Pine Needle Oil	0.1 mL
Glycerin	150 g
Purified Water	830 mL

Dissolve the ephedrine sulfate in the purified water and add the glycerin tragacanth and then the remaining ingredients. Mix well and keep in a closed container for 1 week, stirring occasionally.

In order to prepare uniform gels, it is necessary to disperse the gelling agent in such a manner that it does not form clumps upon the addition of water. Some techniques include the addition of a small quantity of dispersing agent such as alcohol or glycerin and trituration. Another technique is to sprinkle the gelling agent into a vortex of stirred water. If there are a number of other powders in the preparation, the gelling agent first may be triturated with these powders, followed by the addition of water. Shaking the material in a bottle, mixing in a mortar with a pestle or using a mechanical stirrer also are employed. Specific information on the gelling agents is useful in preparing the gels, as described by Zatz and Kushla.³⁵

Gels have been prepared in adhesive form in order to increase the contact time of the active ingredients, such as

insulin with the oral and nasal mucosa, leading to a decrease in plasma glucose. This system also has been investigated as a vaginal dosage form for cervical cancer and a topical dosage form for aphthous stomatitis.

Preservatives should be incorporated into the gels, especially those prepared from natural sources. Appropriate preservatives, depending upon use and the gelling agent, include the parabens at about 0.2%, benzoic acid 0.2% (if the product is acidic) and chlorocresol 0.1%.

The preparation of a few gel bases is given below:

Sodium Alginate Gel Base

Sodium Alginate	2–10 g
Glycerin	2–10 g
Methyl Hydroxybenzoate	0.2 g
a soluble calcium salt (calcium or gluconate)	0.5 g
Purified Water, to make	100 mL

The sodium alginate is wetted in a mortar with glycerin, which aids the dispersion. The preservative is dissolved in about 80 mL of water with the aid of heat, allowed to cool and the calcium salt added, which will increase the viscosity of the preparation. This solution is stirred in a high speed stirrer and the sodium alginate-glycerin mixture added slowly while stirring, until the preparation is homogeneous. The preparation should be stored in a tightly sealed container in a wide mouth jar or tube.

Carbomer Jelly

Carbopol 934	2 g
Triethanolamine	1.65 mL
Parabens	0.2 g
Purified Water, to make	100 mL

The parabens are dissolved in 95 mL of water with the aid of heat and allowed to cool. The Carbopol 934, a commercial grade of carbomer, is added in small amounts to the solution using a high speed stirrer and, after a smooth dispersion is obtained, the preparation is allowed to stand permitting entrapped air to separate. Then the gelling agent, triethanolamine, is added, dropwise, stirring with a plastic spatula to avoid entrapping air and the remaining water incorporated. Other concentrations of carbomer can be used to prepare gels, creams or suspensions.

Gels may contract on standing and some of the solvent then is squeezed out. This process is called *syneresis* and will present a problem in the long-term stability of gels. The addition of relatively large quantities of salts may cause a salting-out of polymers, especially those of an ionic nature. The effect of increasing the temperature may cause rigid gels to melt. An example of an exception to this phenomenon is the gelification of methylcellulose which gels as the temperature rises above $\approx 50^\circ$. This phenomenon is called *thermal gelation*, as described by Schott.³³ In order to minimize water loss from single-phase gels, humectants such as propylene glycol, glycerin or sorbitol are added.

Lotions

Lotions are not defined specifically in the USP, but the BP provides a definition which is broad in nature and indicates that lotions are either liquid or semiliquid preparations which contain one or more active ingredients in an appropriate vehicle. Lotions may contain antimicrobial preservatives and other appropriate excipients such as stabilizers. Lotions are intended to be applied to the unbroken skin without friction. Lotions are usually suspensions of solids in an aqueous medium. A few lotions are, in fact, emulsions or solutions.

Even though lotions usually are applied without friction the insoluble matter should be divided very finely. Particles approaching colloidal dimensions are more soothing to inflamed areas and effective in contact with infected surfaces. A wide variety of ingredients may be added to the preparation to produce better dispersions or to accentuate its cooling, soothing, drying or protective properties. Bentonite is a good example of a suspending agent used in the preparation of lotions. Methylcellulose or sodium carboxymethylcellulose, eg, will localize and hold the active ingredient in contact with

the a
wate
mois
cooli
form
De
esth
prot
prev
Antil
fur,
flow
mon
aspe
com
The
of th
lent
Exte
assis
Lo
smo
trit
ter d
large
sic e
pow
susp
face
Th
Loti

Ca
Zin
Gly
Be
Ca

Dil
topic
100 r
form
Final
and s
If
benu

M
has
ing
colle
Fo
line
than

Ca
Zin
Gly
Av
Ca
Ca

Mi
This
mine
carb
maki

W
are
tion

the affected site and at the same time be rinsed off easily with water. A formulation containing glycerin will keep the skin moist for a considerable period of time. The drying and cooling effect may be accentuated by adding alcohol to the formula.

Dermatologists frequently prescribe lotions containing anesthetics, antipruritics, antiseptics, astringents, germicides, protectives or screening agents, to be used in treating or preventing various types of skin diseases and dermatitis. Antihistamines, benzocaine, calamine, resorcin, steroids, sulfur, zinc oxide, betamethasone derivatives, salicylic acid, safflower oil, minoxidil and zirconium oxide are ingredients common in unofficial lotions. In many instances the cosmetic aspects of the lotion are of great importance. Many lotions compare badly with cosmetic preparations of a similar nature. The manufacture of fine lotions to meet the specialized needs of the dermatologist provides the pharmacist with an excellent opportunity to demonstrate professional competence. Extensive studies on lotions, as described by Harb,³⁶ will assist the pharmacist to attain this goal.

Lotions may be prepared by triturating the ingredients to a smooth paste and then adding the remaining liquid phase with trituration. High-speed mixers or colloid mills produce better dispersions and, therefore, are used in the preparation of larger quantities of lotion. Calamine Lotion USP is the classic example of this type of preparation and consists of finely powdered, insoluble solids held in more or less permanent suspension by the presence of suspending agents and/or surface-active agents.

The formula and the method of preparation of Calamine Lotion USP is given

Calamine Lotion

Calamine	80 g
Zinc Oxide	80 g
Glycerin	20 mL
Bentonite Magma	250 mL
Calcium Hydroxide Topical Solution, a sufficient quantity, to make	1000 mL

Dilute the bentonite magma with an equal volume of calcium hydroxide topical solution. Mix the powder intimately with the glycerin and about 100 mL of the diluted magma, triturating until a smooth, uniform paste is formed. Gradually incorporate the remainder of the diluted magma. Finally add enough calcium hydroxide topical solution to make 1000 mL and shake well.

If a more viscous consistency in the Lotion is desired, the quantity of bentonite magma may be increased to not more than 400 mL.

Many investigators have studied Calamine Lotion and this has led to the publication of many formulations, each possessing certain advantages over the others but none satisfying the collective needs of all dermatologists.

Formulations containing Avicel R (hydrated microcrystalline cellulose, FMC) and carboxymethylcellulose settle less than the official preparations.

Calamine Lotion

Calamine	8 g
Zinc Oxide	8 g
Glycerin	2 mL
Avicel R Gel	2 g
Carboxymethylcellulose	2 g
Calcium Hydroxide Solution, a sufficient quantity, to make	100 mL

Mix 45 g of Avicel R with 55 g of water with a suitable electric mixer. This gel is used in the preparation of the calamine lotion. Mix the calamine and the zinc oxide with the glycerin, the gel and the carboxymethylcellulose. Add sufficient calcium hydroxide solution to make the product measure 100 mL.

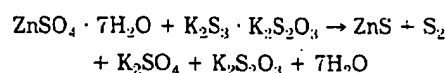
While most lotions are prepared by trituration, some lotions are formed by chemical interaction in the liquid. White Lotion is an example.

White Lotion

Zinc Sulfate	40 g
Sulfurated Potash	40 g
Purified Water, a sufficient quantity to make	1000 mL

Dissolve the zinc sulfate and the sulfurated potash separately, each in 450 mL of purified water and filter each solution. Add slowly the sulfurated potash solution to the zinc sulfate solution with constant stirring. Then add the required amount of purified water, and mix.

Sulfurated potash is a solid of variable composition but usually is described as $K_2S_3 \cdot K_2S_2O_3$. The chemical reaction which occurs when sulfurated potash solution is added to the zinc sulfate is



This lotion must be prepared fresh and does not contain a suspending agent. Bentonite Magma has been used in some formulations. Coffman and Huyck³⁷ include a detailed discussion of the chemistry and the problems involved in the preparation of a suitable product.

An example of a lotion that is an emulsion is Benzyl Benzoate Lotion USP. The formula and method of preparation are as follows:

Benzyl Benzoate	250 mL
Triethanolamine	5 g
Oleic Acid	20 g
Purified Water	750 mL
To make about	1000 mL

Mix the triethanolamine with the oleic acid, add the benzyl benzoate, and mix. Transfer the mixture to a suitable container of about 2000-mL capacity, add 250 mL of purified water, and shake the mixture thoroughly. Finally add the remaining purified water, and again shake thoroughly.

The triethanolamine forms a soap with the oleic acid and functions as the emulsifying agent to form a stable product. This type of emulsifying agent is almost neutral in water and gives a pH of about 8 and thus should not irritate the skin.⁶ An example of the wide variety of formulations of benzyl benzoate is provided by Bhargava and Nicolai.³⁸

Some lotions are clear solutions as exemplified by Amino-benzoic Acid Lotion BP.

Aminobenzoic Acid	50 g
Glycerol	200 mL
Ethanol 96%	600 mL
Purified Water freshly boiled and cooled, sufficient to produce	1000 mL

Dissolve the aminobenzoic acid in the ethanol 96%, add the glycerol and sufficient purified water to produce 1000 mL and mix. The ethanol is used to dissolve the aminobenzoic acid and provide a cooling effect. The glycerol (glycerin) is used for its emollient effect. Since lotions may be solutions, suspensions or emulsions, the method of preparation is similar to those types of formulations described above.

Several lotions are listed in the USP and contain, for example, antibiotics, steroids, scabicides and sunscreens.

A formula for hydrocortisone lotion is given in the PC.

Hydrocortisone Lotion

Hydrocortisone, in ultrafine powder	10.0 g
Chlorocresol	0.5 g
Self-emulsifying monostearin	40.0 g
Glycerol	63.0 g
Purified water, freshly boiled and cooled to make	1000.0 g

To prepare the base, the chlorocresol is dissolved in 850 mL of water with the aid of gentle heat, the self-emulsifying monostearin is added and the mixture heated to 60° with stirring until completely dispersed. The hydrocortisone is triturated with the glycerol and the trituration is then incorporated, with stirring, into the warm base, allowed to cool while stirring, then add the remainder of the water and mix.

Certain lotions tend to separate or stratify on long standing, and they require a label directing that they be shaken well before each use. All lotions should be labeled "For External Use Only."

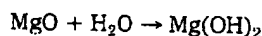
Microorganisms may grow in certain lotions if no preservative is included. Care should be taken to avoid contaminating the lotion during preparation, even if a preservative is present.

Magnas and Milks

Magnas and milks are aqueous suspensions of insoluble, inorganic drugs and differ from gels mainly in that the suspended particles are larger. When prepared, they are thick and viscous and, because of this, there is no need to add a suspending agent.

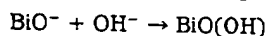
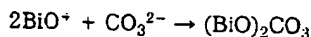
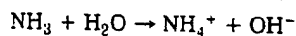
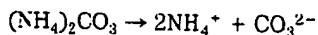
Bentonite Magma USP is prepared by simple hydration. Two procedures are given for the preparation of this product, and these are described in Chapter 80. Dihydroxyaluminum Aminoacetate Magma is the other magma in the USP.

Milk of Magnesia USP is a suspension of magnesium hydroxide containing not less than 80 mg of $Mg(OH)_2$ per mL. The specifications for double strength or triple strength are that these products should contain not less than 160 mg or 240 mg of $Mg(OH)_2$ per mL, respectively. It has an unpleasant, alkaline taste which can be masked with 0.1% citric acid (to reduce alkalinity) and 0.05% of a volatile oil or a blend of volatile oils. Magnesium hydroxide is prepared by the hydration of magnesium oxide.



Milk of Bismuth USP contains bismuth hydroxide and bismuth subcarbonate in suspension in water. The Magma is prepared by reacting bismuth subnitrate with nitric acid and ammonium carbonate with ammonia solution and then mixing the resulting two solutions.

The following reactions occur during the preparation of the magma.



If the insoluble substance is precipitated fresh by mixing hot, dilute solutions, there is only slight sedimentation on standing. This characteristic of milks or magnas sometimes is enhanced by passing the product through a colloid mill.

For the most part, magnas and milks are intended for internal use, eg, Milk of Magnesia USP and Dihydroxy Aluminum Aminoacetate Magma USP, although Bentonite Magma is used primarily as a suspending agent for insoluble substances for local application and occasionally for internal use. All magnas require a "Shake Well" label. Freezing must be avoided.

Several antimicrobial preservatives have been tested in liquid antacid preparations for their stability and effectiveness, such as benzoic acid, chlorhexidine, methylparaben, propylparaben, sorbic acid, propylene glycol or ethanol. It was found that a combination of methylparaben and sorbic acid was superior to the parabens alone.

Mixtures

The USP does not recognize the term mixture; however, the BP defines the term as

Mixtures are oral liquids containing one or more active ingredients, dissolved, suspended or dispersed in a suitable vehicle. Suspended solids may separate slowly on standing, but are easily redispersed on shaking.

The insoluble substance usually does not make the mixture very viscous, and the particles may be held in suspension by using suitable suspending or thickening agents. This class was introduced originally to secure uniformity in the formulas of certain well-known and largely used preparations. Frequently, the term *mixture* is applied loosely to aqueous preparations of every description. The term *shake mixture* is used often for liquid preparations which contain insoluble ingredients and, therefore, must be shaken before use. The

term *suspension* is used to describe a number of similar preparations.

The following is a formula for a mixture in the BP, which is a solution for an extemporaneous preparation.

Ammonium Chloride Mixture

Ammonium Chloride	100 g
Aromatic Ammonia Solution	50 mL
Liquorice Liquid Extract	100 mL
Water for Preparations to	1000 mL

It should be prepared recently.

The following mixture is an example of a suspension and is used for the treatment of diarrhea. The pectin and the tragacanth in Kaolin Mixture with Pectin act as suspending agents. An alternate formula, based on Veegum (*Vanderbilt*) and sodium carboxymethylcellulose, has been proposed by Kalish.³⁹

Kaolin Mixture with Pectin

Veegum	0.88 g
Sodium Carboxymethylcellulose	0.22 g
Purified Water	79.12 g
Kaolin	17.50 g
Pectin	0.44 g
Saccharin	0.09 g
Glycerin	1.75 g

Add the Veegum and the sodium carboxymethylcellulose to the water with continuous stirring. Add, with mixing, the kaolin. Mix the pectin, saccharin and glycerin and add to the suspension. A preservative and flavoring agent may be added to the product.

The insoluble material in mixtures must be in a very finely divided state and uniformly distributed throughout the preparation. This is accomplished with colloid mills, special methods of precipitation and suspending agents. There are three main reasons for having the insoluble substances in as fine a state of subdivision as possible.

1. The more nearly the colloidal state is approached by protectives, such as kaolin, magnesium trisilicate or magnesium phosphate, the more active they become as adsorbents and protectives when in contact with inflamed surfaces.

2. Finely divided particles are suspended more readily and settle out much more slowly than large particles, thus enabling the patient to obtain uniform doses of suspended substances. Homogeneous mixtures are desirable especially when administering medication to form an evenly distributed, protective coating on the gastrointestinal tract.

3. The palatability of many preparations is enhanced by the use of colloidal suspending agents.

Mixtures containing suspended material should have a "Shake Well" label affixed to the container in which they are dispensed.

Mixtures, including suspensions, are subject to contamination by microorganisms that remain viable and are a potential health hazard during the period of use of the products. Survival times of organisms depend on the preservative used. A kaolin pediatric mixture that contains benzoic acid kills organisms rapidly, whereas organisms survived for more than a week in a magnesium trisilicate mixture that contained no more than a trace of peppermint oil, as noted by Westwood.⁴⁰

Official Suspensions

The USP places particular emphasis on the term *suspension* by providing specific definitions for a variety of oral, parenteral and ophthalmic preparations formulated in such a way that an insoluble substance is suspended in a liquid at some stage of the manufacturing or dispensing process. The USP definition begins as follows:

Suspensions are liquid preparations which consist of solid particles dispersed throughout a liquid phase in which the particles are not soluble. Dosage forms officially categorized as Suspensions are designated as such if they are not included in other more specific categories of suspensions, such as Oral Suspensions, Topical Suspensions, etc. (see these other categories). Some suspensions are prepared and ready for use, while

others are prepared as solid mixtures intended for constitution just before use with an appropriate vehicle. Such products are designated "for Oral Suspension," etc.

This definition relates the term suspension to milks, magmas and lotions which have been described above.

While there are a number of monographs dealing with suspensions in the USP, neither the definition nor the monographs give specific directions for the preparation of the suspension, although pharmacopeias usually permit the addition of suitable flavoring agents, suspending agents, preservatives and certified color additives. One procedure for the preparation of the commonly used Trisulfapyrimidines Oral Suspension is given below.

Trisulfapyrimidines Oral Suspension

Veegum	1.00 g
Syrup USP	90.60 g
Sodium Citrate	0.78 g
Sulfadiazine	2.54 g
Sulfamerazine	2.54 g
Sulfamethazine	2.54 g

Add the Veegum, slowly and with continuous stirring, to the syrup. Incorporate the sodium citrate into the Veegum-syrup mixture. Premix the sulfa drugs, add to the syrup, stir and homogenize. Add sufficient 5%

citric acid to adjust the pH of the product to 5.6. A preservative and a flavoring agent may be added to the product.

Methods of preparation for those formulations which contain several active ingredients and are produced in large quantities tend to be more complex than that given above and are described previously.

Many formulations for suspensions are given in the BP and the PC under *Mixtures*.

A properly prepared suspension has a number of desirable properties:

1. The suspended material should not settle rapidly.
2. Particles that do settle should not form a hard cake and easily should be resuspended uniformly on shaking.
3. The suspension should pour freely from the container.

Insoluble powders that do not disperse evenly throughout the suspending medium, when shaken, should be powdered finely and levigated with a small amount of an agent such as glycerin, alcohol or a portion of the dispersion of the suspending agent. The other ingredients are incorporated and the remainder of the dispersion of the suspending agent is incorporated gradually by trituration to produce the appropriate volume.

Suspensions intended for parenteral or ophthalmic use also are described in the USP. For a discussion of these suspensions, see Chapter 87 and 89.

Extracts

Extraction

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures.

The products so obtained from plants are relatively impure liquids, semisolids or powders intended only for oral or external use. These include classes of preparations known as decoctions, infusions, fluidextracts, tinctures, pilular (semisolid) extracts and powdered extracts. Such preparations popularly have been called galenicals, after Galen, the 2nd century Greek physician. For additional information concerning extraction and extractives, see RPS 15, Chapter 86.

Extraction continues to be of considerable interest in order to obtain improved yields of drugs derived from plant and animal sources. For example, improved extraction of digitalis glycosides has been carried out using a pulsating, perforated, bottom column. Other techniques include ultrasonics, rotary-film evaporators, liquid and supercritical carbon dioxide, hydrodistillation, liquid chromatography, multiple-solvent extraction, countercurrent extraction and gravitation dynamics.

This discussion is concerned primarily with basic extraction procedures for crude drugs to obtain the therapeutically desirable portion and eliminate the inert material by treatment with a selective solvent, known as the menstruum. Extraction differs from solution in that the presence of insoluble matter is implied in the former process. The principal methods of extraction are maceration, percolation, digestion, infusion and decoction. The quality of the finished product can be enhanced by standardizing primary extracts and carrying out analytical assays during production on the raw materials, intermediate products and manufacturing procedures.

The processes of particular importance, insofar as the USP is concerned, are those of maceration and percolation, as described specifically for Belladonna Extract USP and Cascara Sagrada Extract USP. Most pharmacopeias refer to such processes for extraction of active principles from crude drugs. The USP provides general directions for both maceration and percolation under the heading of *Tinctures*.

Maceration—In this process the solid ingredients are placed in a stoppered container with 750 mL of the prescribed solvent and allowed to stand for a period of at least 3 days in a warm place with frequent agitation, until soluble matter is dissolved. The mixture is filtered and, after most of the liquid has drained, the residue on the filter is washed with sufficient

quantity of the prescribed solvent or solvent mixture; the filtrates are combined to produce 1000 mL.

Percolation—The ground drug is mixed with the appropriate quantity of the prescribed solvent to make it evenly and uniformly damp. It is allowed to stand for 15 minutes, then transferred to a percolator (a narrow coned-shaped vessel, open at both ends). Sufficient prescribed solvent is added to saturate the drug. The top is placed on the percolator and, when the liquid is about to drip from the apparatus, the lower opening is closed. The drug is allowed to macerate for 24 hours or for the specified time. If no assay is directed, the percolation is allowed to proceed slowly or at the specified rate gradually adding sufficient solvent to produce 1000 mL of solution. If an assay is required, only 950 mL of percolate are collected and mixed and a portion assayed as directed. The rest of the percolate is diluted with the solvent to produce a solution that conforms to the required standard and then mixed.

Digestion—This is a form of maceration in which *gentle heat* is used during the process of extraction. It is used when moderately elevated temperature is not objectionable and the solvent efficiency of the menstruum is increased thereby.

Infusion—An infusion is a dilute solution of the readily soluble constituents of crude drugs. Fresh infusions are prepared by macerating the drugs for a short period of time with either cold or boiling water. US official compendia have not included infusions for some time. An example is Concentrated Compound Gentian Infusion BP 1973.

Decoction—This once-popular process extracts water-soluble and heat-stable constituents from crude drugs by boiling in water for 15 min, cooling, straining and passing sufficient cold water through the drug to produce the required volume.

Extractive Preparations

After a solution of the active constituents of a crude drug is obtained by maceration or percolation, it may be ready for use as a medicinal agent, as with certain tinctures or fluidextracts, or it may be processed further to produce a solid or semisolid extract.

For a discussion of *resins* and *oleoresins* obtained by solvent extraction of plant exudates see Chapter 26, under *Plant Exudates*.

Tinctures—Tinctures are defined in the USP as being alcoholic or hydroalcoholic solutions prepared from vegetable materials or from chemical substances, an example of the latter being Iodine Tincture. Traditionally, tinctures of potent vegetable drugs essentially represent the activity of 10 g of the drug in each 100 mL of tincture, the potency being

adjusted following assay: Most other tinctures of vegetable drugs represent the extractive from 20 g of the drug in 100 mL of tincture.

The USP specifically describes two general processes for preparing tinctures, one by percolation designated as Process P, and the other by maceration, as Process M. These utilize the methods described under *Extraction*.

Process P includes a modification so that tinctures that require assay for adjustment to specified potency thus may be tested before dilution to final volume. A tincture prepared by Process P as modified for assayed tinctures is Belladonna Tincture.

Examples of tinctures prepared by Process M are Compound Benzoin Tincture USP and Sweet Orange Peel Tincture USP XXI (the latter contains the extractive from 50 g of sweet orange peel in 100 mL of tincture).

Fluidextracts—The USP defines fluidextracts as being liquid preparations of vegetable drugs, containing alcohol as a solvent or as a preservative, or both, so made that, in general, each mL contains the therapeutic constituents of 1 g of the standard drug that it represents. While the USP states that pharmacopoeial fluidextracts are made by percolation, the official compendia previously have described general procedures for three percolation methods used in making fluidextracts.

Process A is a percolation method that can be modified for fluidextracts that must be assayed.

Process E is an alternative for Process A in which percolation is conducted on a column of drug much greater in length than in diameter.

Process D is used for preparing fluidextracts with boiling water as the menstruum, alcohol being added as a preservative to the concentrated percolate; this is the procedure used for preparing Cascara Sagrada Fluidextract USP XXI.

The BP and PC use the designation *Liquid Extracts* for fluidextracts.

Extracts—Extracts are defined in the USP as concentrated preparations of vegetable or animal drugs obtained by removal of the active constituents of the respective drugs with suitable menstrua, evaporation of all or nearly all of the solvent and adjustment of the residual masses or powders to the prescribed standards.

Three forms of extracts were recognized in USP XXI: semiliquids or liquids of syrupy consistency, plastic masses (known as *pilular* or *solid extracts*) and dry powders (known as *powdered extracts*). Extracts, as concentrated forms of the drugs from which they are prepared, are used in a variety of solid or semisolid dosage forms. The USP states that pilular extracts and powdered extracts of any one drug are interchangeable medicinally, but each has its own pharmaceutical advantages. Pilular extracts, so-called because they are of a consistency to be used in pill masses and made into pills and are suited especially for use in ointments and suppositories. Powdered extracts are suited better for incorporation into a dry formulation, as in capsules, powders or tablets. Semiliquid extracts, or extracts of a syrupy consistency, may be used in the manufacture of some pharmaceutical preparations.

Most extracts are prepared by extracting the drug by percolation. The percolate is concentrated, generally by distillation under reduced pressure. The use of heat is avoided where possible because of potential injurious effect on active constituents. Powdered extracts which are made from drugs that contain inactive oily or fatty matter may have to be defatted or prepared from defatted drug. For diluents that may be used to adjust an extract to prescribed standards, see the USP XXI.

Pure Glycyrrhiza Extract [USP XXI] is an example of a pilular extract; Belladonna Extract USP and Hyoscyamus Extract PC are examples of powdered extracts (the former is prepared also as a pilular extract and the latter as a liquid extract).

References

1. Connors KA, Amidon GL, Kennon L: *Chemical Stability of Pharmaceuticals*, Wiley, New York, 80, 1979.

2. Kurup TRR, Wan LCS: *Pharm J* 237: 761, 1986.
3. Bruch CW: *Drug Cosmet Ind* 111(4): 51, 1972.
4. Coates D: *Mfg Chem Aerosol News* 44(6): 35, (8): 41, (10): 34, (12): 19, 1973; 45(1): 19, 1974.
5. Reynolds JEF, ed: *Martindale, The Extra Pharmacopoeia*, 30th ed, The Pharmaceutical Press, London, 1132, 1993.
6. *Handbook of Pharmaceutical Excipients*, APhA & Pharmaceutical Society of Great Britain, Washington DC & London, 4, 1986.
7. Hickman K et al: *Science* 180: 15, 1973.
8. Montgomery HA et al: *Am J Hosp Pharm* 43: 118, 1986.
9. Tricca RE: *Drug Cosmet Ind* 142(5): 32, 1988.
10. Bergen A: *Can J Hosp Pharm* 30(4): 109, 1977.
11. Meer T: *Flavoring Pharmaceutical Preparations* (SK&F Selected Pharm Res Refs No 4), SmithKline, Philadelphia, Feb 11, 1957.
12. Schumacher GE: *Am J Hosp Pharm* 24: 588, 713, 1967; 25: 154, 1968.
13. *Am Drug* 175(5): 24, 1977.
14. Ryder J: *Int J Pharm Technol Prod Mfg* 1(1): 14, 1979.
15. *The Chemistry and Rheology of Water-Soluble Gums and Colloids* (Monograph 24), Society of Chemical Industry, London, 1966.
16. Sumner ED: *JAPhA NS8*: 250, 1968.
17. Rettig H: *Acta Pharm Technol* 24: 143, 1978 through *Int Pharm Abstr* 19: 5096, 1982.
18. White RF: *Pharmaceutical Emulsions and Emulsifying Agents*, 4th ed, Chemist & Druggist, London, 1964.
19. Griffin WC, Lynch MJ, Lathrop LB: *Drug Cosmet Ind* 101(4): 41, (5): 52, 1967.
20. Rieger MM. In Lachman L, Lieberman HA, Kanig JL, eds: *The Theory and Practice of Industrial Pharmacy*, 3rd ed, Lea & Febiger, Philadelphia, Chap 17, 1986.
21. Florence AT, Whitehill D: *Int J Pharm* 11: 277, 1982.
22. Prince LM, ed: *Microemulsions: Theory and Practice*, Academic, New York, 1977.
23. Rosano HL et al: *J Soc Cosmet Chem* 39: 201, 1988.
24. Lin TJ: *Ibid* 29: 117, 1978; 35: 357, 1984.
25. Eisberg N: *Mfg Chem* 53(1): 27, 1982.
26. Fox C. In Breu MM, ed: *Cosmetic Science*, vol 2, Academic, New York, Chap 1, 1980.
27. Scott RR. In Lieberman HA, Rieger MM, Banker GS, eds: *Pharmaceutical Dosage Forms: Disperse Systems*, vol 2, Dekker, New York, Chap 1, 1989.
28. Parrot EL. In Lachman L, Lieberman HA, Kanig JL, eds: *Theory and Practice of Industrial Pharmacy*, 3rd ed, Lea & Febiger, Philadelphia, Chap 2, 1986.
29. Nash RA. In Lieberman HA, Rieger MM, Banker GS, eds: *Pharmaceutical Dosage Forms: Disperse Systems*, vol 1, Dekker, New York, Chap 5, 1989.
30. Reynolds JEF, ed: *Martindale, The Extra Pharmacopoeia*, 28th ed, Pharmaceutical Press, London, 947, 1982.
31. Tortorici MP: *Am J Hosp Pharm* 36: 22, 1979.
32. Scheer AJ: *Drug Cosmet Ind* 128(4): 40, (5): 39, (6): 52, 1981.
33. Schott H. In Martin A, Swarbrick J, Cammarata A, eds: *Physical Pharmacy*, Lea & Febiger, Philadelphia, Chap 22, 1983.
34. Collett DM. In Collett DM, Aulton ME, eds: *Pharmaceutical Practice*, Churchill Livingstone, Edinburgh, Chap 14, 1990.
35. Zauz JL, Kushla GP. In Lieberman HA, Rieger MM, Banker GS, eds: *Pharmaceutical Dosage Forms: Disperse Systems*, vol 2, Dekker, New York, Chap 13, 1989.
36. Harb NA: *Cosmet Perfum* 89(4): 67, 1974; Shapiro WB: *Cosmet Toiletries* 97(3): 27, 1982.
37. Coffman HL, Huyck CL: *Am J Hosp Pharm* 20: 132, 1963.
38. Bhargava HN, Nicolai DW. In Lieberman HA, Rieger MM, Banker GS, eds: *Pharmaceutical Dosage Forms: Disperse Systems*, vol 2, Dekker, New York, Chap 7, 1989.
39. Kalish J: *Drug Cosmet Ind* 94: 276, 1964.
40. Westwood N: *Pharm J* 208: 153, 1972.

Bibliography

General

- Lieberman HA, Rieger MM, Banker GS, eds: *Pharmaceutical Dosage Forms: Disperse Systems*, vol 1 & 2, Dekker, New York, 1989.

Emulsions

- Becher P: *Emulsions: Theory & Practice*, 2nd ed, Reinhold, New York, 1965.
- Griffin WC, Lynch MJ, Lathrop LB: *Drug Cosmet Ind* 101(4): 41, (5): 52, 1967.

Spal
fy
Equ
Buss
Fox
19
Lagn
Olds
Rees
Scot
Ph
Ne
Wasl

Spalton LM. In White RF, ed: *Pharmaceutical Emulsions and Emulsifying Agents*, 4th ed, Chemist & Druggist, London, 1964.

Equipment

Busse DJ: *Mfg Chem* 61(7): 39, 1990.

Fox C. In Breuer MM, ed: *Cosmetic Science*, vol 2, Academic, New York, 1980.

Lagman B: *Drug Develop Ind Pharm* 14(18): 2705, 1988.

Oldshue JY: *Fluid Mixing Technology*, McGraw Hill, New York, 1983.

Rees LH: *Drug Cosmet Ind* 101(5): 102, 1967.

Scott RR. In Lieberman HA, Rieger MM, Banker GS, eds: *Pharmaceutical Dosage Forms: Disperse Systems*, vol 2, Dekker, New York, 1989.

Washington C: *Mfg Chem* 59(3): 49, 1988.

Excipient Properties

Handbook of Pharmaceutical Excipients, APHA & Pharmaceutical Society of Great Britain, Washington DC & London, 1986.

Reynolds JEF, ed: *Martindale, The Extra Pharmacopoeia*, 29th ed, Pharmaceutical Press, London, 1989.

Solutions

Ryder J: *Int J Pharm Tech Prod Mfg* 1(1): 14, 1979.

Suspensions

Plaizier-Vercammen JA, Janssens E: *Labo-Pharma—Probl Tech* 32(345, Sep): 583, 1984.

Scheer AJ: *Drug Cosmet Ind* 128(4): 40 (5): 39; (6): 52, 1981.

CHAPTER 87

Parenteral Preparations

Kenneth E. Avis, DSc

Emeritus Professor, Pharmaceutics
College of Pharmacy
University of Tennessee, Memphis
The Health Science Center
Memphis, TN 38163

John W. Levchuk, PhD

Adjunct Associate Professor of Pharmaceutics
School of Pharmacy
University of Maryland
Baltimore, MD 21201

The distinctive characteristics of parenteral (Gk, *para*—*en*—*teron*, beside the intestine) dosage forms of drugs will be discussed in this chapter. These dosage forms differ from all other drug dosage forms because of the unique requirements imposed because they are injected directly into body tissue through the primary protective system of the human body, the skin and mucous membranes. Therefore, they must be exceptionally pure and free from physical, chemical and biological contaminants. These requirements place a heavy responsibility on the pharmaceutical industry to practice good manufacturing practices (GMPs) in the manufacture of parenteral dosage forms and upon pharmacists to practice good aseptic practices (GAPs) in dispensing them for administration to patients.

Many of the newer drugs, particularly these derived from the new developments in biotechnology, can only be given parenterally because they are inactivated in the gastrointestinal tract, when given by mouth. Further, the potency and specificity of many of these drugs requires strict control of their administration to the patient. A parenteral route of administration meets both of these critical requirements.

This chapter will begin with a brief review of the historical events contributing to the development of this distinctive dosage form. Consideration will then be given to some of the distinguishing characteristics of these dosage forms and how they are administered to patients. The majority of the remainder of the chapter will discuss the various factors required for the preparation of a pure, safe and effective parenteral product.

History¹

One of the most significant events in the beginnings of parenteral therapy was the first recorded injection of drugs into the veins of living animals, in about 1657, by the architect Sir Christopher Wren. From such a very crude beginning, the technique for intravenous injection and knowledge of the implications therefore developed slowly during the next century and a half. In 1855 Dr Alexander Wood of Edinburgh described what was probably the first subcutaneous injection of drugs for therapeutic purposes using a true hypodermic syringe.

The latter half of the 19th century brought increasing concern for safety in the administration of parenteral solutions, largely because of the work of Robert Koch and Luis Pasteur. While Charles Chamberland was developing both hot-air and steam sterilization techniques and the first bacteria-retaining filter (made of unglazed porcelain), Stanislaus Limousin was developing a suitable container, the all-glass ampul. In the middle 1920s Dr Florence Seibert provided proof that the disturbing chills and fever which often followed the intravenous injection of drugs was caused by potent products of microbial growth, pyrogens, which could be eliminated from water by distillation and from glassware by heating at elevated temperatures.

Of the technical developments that have contributed to the high quality standards currently achievable in the preparation of parenteral dosage forms, the two that have probably contrib-

uted most are the development of HEPA-filtered laminar airflow and the development of membrane microfiltration for solutions. The former made it possible to achieve ultraclean environmental conditions for processing from solutions by filtration both viable and nonviable particles of microbial size and smaller. However, many other developments in recent years have produced an impressive advance in the technology associated with the safe and reliable preparation of parenteral dosage forms. The following list identifies a few of the events which have contributed to that development.

1926—Parenterals were accepted for inclusion in the fifth edition of the *National Formulary*.

1933—The practical application of freeze-drying to clinical materials was accomplished by a team of scientists at the University of Pennsylvania.

1938—The Food, Drug and Cosmetic Act was passed by Congress, establishing the Food and Drug Administration (FDA).

1944—The sterilant ethylene oxide was discovered.

1946—The Parenteral Drug Association was organized.

1961—The concept of laminar airflow was developed by WJ Whitfield.

1962—The FDA was authorized by Congress to establish current good manufacturing practice (CGMP or GMP) regulations.

1965—Total parenteral nutrition (TPN) was developed by SJ Dudrick.

1972—The Limulus Amebocyte Lysate test for pyrogens in parenteral products was developed by JF Cooper.

1974—The concept of validation of processes used in the manufacture of parenteral products was introduced by the FDA.

1977—The principles for clean-in-place (CIP) and steam-in-place (SIP) were introduced.

Early 1980s—Home Health Care emerged as an alternative for patients whose health status permitted release from a hospital to care in the home environment.

1982—Insulin, derived through the new discipline of biotechnology, ushered in the drug class of polypeptides with their inherent stability challenges for parenteral dosage-form development.

1987—Parametric release was accepted by the FDA for selected products terminally sterilized by a validated heat process.

The FDA published *Guideline on Sterile Products Produced by Aseptic Processing*, one of several nonregulatory publications to help industry know what the FDA considers to be acceptable.

Late 1980s—The development of computer capabilities has led to the automation of many process operations and to a revolution in documentation and recordkeeping.

1991—The FDA proposed requiring manufacturers to use a terminal sterilization process when preparing a sterile drug product unless such a process adversely affects the drug product.

Administration

Injections may be classified in six general categories:

1. Solutions ready for injection.
2. Dry, soluble products ready to be combined with a solvent just prior to use.
3. Suspensions ready for injection.
4. Dry, insoluble products ready to be combined with a vehicle just prior to use.
5. Emulsions.
6. Liquid concentrates ready for dilution prior to administration.

These injections may be administered by such routes as intravenous, subcutaneous, intradermal, intramuscular, intra-

artic
term
emp.
will
susp
strea
capil
quir
tion
area
for i
mini
the
subs
W
sess
need
nous
form
the
tic r
adm
are
tere
expe
adm
they
unco
or la
vant
adm
the
corr
tion.
ately
One
tion
ally
adve
insti
for t

Par

D
(LV)
is co
sma
otic.
inje
prof
as is
Join
tion
trou
ties
ents
pati
othe
Ti
tion
con
eval
upd
and
gain
on t
A
beer
fact
stitu
pote
may
Ulti

articular and intrathecal. The nature of the product will determine the particular route of administration that may be employed. Conversely, the desired route of administration will place requirements on the formulation. For example, suspensions would not be administered directly into the blood stream because of the danger of insoluble particles blocking capillaries. Solutions to be administered subcutaneously require strict attention to tonicity adjustment, otherwise irritation of the plentiful supply of nerve endings in this anatomical area would give rise to pronounced pain. Injections intended for intraocular, intraspinal, intracisternal and intrathecal administration require the highest purity standards because of the sensitivity of tissues encountered to irritant and toxic substances.

When compared with other dosage forms, injections possess select advantages. If immediate physiological action is needed from a drug, it usually can be provided by the intravenous injection of an aqueous solution. Modification of the formulation or another route of injection can be used to slow the onset and prolong the action of the drug. The therapeutic response of a drug is controlled more readily by parenteral administration since the irregularities of intestinal absorption are circumvented. Also, since the drug normally is administered by a professionally trained person, it confidently may be expected that the dose was actually and accurately administered. Drugs can be administered parenterally when they cannot be given orally because of the unconscious or uncooperative state of the patient, or because of inactivation or lack of absorption in the intestinal tract. Among the disadvantages of this dosage form are the requirement of asepsis at administration, the risk of tissue toxicity from local irritation, the real or psychological pain factor and the difficulty in correcting an error, should one be made. In the latter situation, unless a direct pharmacological antagonist is immediately available, correction of an error may be impossible. One other disadvantage is that daily or frequent administration poses difficulties, either for the patient to visit a professionally trained person or to learn to inject oneself. However, the advent of home health care as an alternative to extended institutional care has mandated the development of programs for training lay persons to administer these dosage forms.

Parenteral Combinations

During the administration of large-volume parenterals (LVPs), such as 1000-mL of 0.9% sodium chloride solution, it is common practice for a physician to order the addition of a small-volume therapeutic parenteral (SVP), such as an antibiotic, to avoid the discomfort for the patient of a separate injection. While the pharmacist is the most qualified health professional to be responsible to prepare such combinations, as is clearly stated in the Hospital Accreditation Manual of the Joint Commission on Accreditation of Healthcare Organizations,² interactions among the combined products can be troublesome even for the pharmacist. In fact, incompatibilities can occur and cause inactivation of one or more ingredients or other undesired reactions. In some instances incompatibilities are visible as precipitation or color change, but in other instances there may be no visible effect.

The many potential combinations present a complex situation even for the pharmacist. To aid in making decisions concerning potential problems, a valuable compilation of relevant data has been assembled by Trissel,³ and is regularly updated. Further, the advent of computerized data storage and retrieval systems has provided a means to organize and gain rapid access to such information. Further information on this subject may be found in Chapter 88.

As studies have been undertaken and more information has been gained, it has been shown that knowledge of variable factors such as pH and the ionic character of the active constituents aids substantially in understanding and predicting potential incompatibilities. Kinetic studies of reaction rates may be used to describe or predict the extent of degradation. Ultimately, a thorough study should be undertaken of each

therapeutic agent in combination with other drugs and IV fluids, not only of generic but of commercial preparations, from the physical, chemical and therapeutic aspects.

Ideally, no parenteral combination should be administered unless it has been studied thoroughly to determine its effect on the therapeutic value and the safety of the combination. However, such an ideal situation may not exist. Nevertheless, it is the responsibility of the pharmacist to be as familiar as possible with the physical, chemical and therapeutic aspects of parenteral combinations and to exercise the best possible judgment as to whether or not the specific combination extemporaneously prescribed is suitable for use in a patient.

General Considerations

An inherent requirement for parenteral preparations is that they be of the very best quality and provide the maximum safety for the patient. Therefore, whether they are prepared from commercially available sterile components, as is usually the case in hospital pharmacies and similar sites, or from nonsterile ingredients in a manufacturing mode, as is the case in the pharmaceutical industry, the persons responsible for their preparation must apply their skills intelligently and diligently. Further, the possession and application of high moral and professional ethics on the part of the persons responsible is the ingredient most vital to achieving the desired quality in the products prepared.

The preparation of parenteral products from sterile components in pharmacies of hospitals and similar sites is discussed further in Chapter 88. In this chapter emphasis will be placed on the preparation of parenteral products from non-sterile components in the highly technologically advanced plants of the pharmaceutical industry, using GMP principles. In the pursuit of GMP, consideration should be given to:

1. Ensure the personnel responsible for assigned duties are capable and qualified to perform them.
2. Ensure that ingredients used in compounding the product have the required identity, quality and purity.
3. Validate critical processes to be sure that the equipment used and the processes followed will ensure that the finished product will have the qualities expected.
4. Maintain a production environment suitable for performing the critical processes required, addressing such matters as orderliness, cleanliness and asepsis.
5. Confirm through adequate quality-control procedures that the finished products have the required potency, purity and quality.
6. Establish through appropriate stability evaluation that the drug products will retain their intended potency, purity and quality until the established expiration date.
7. Ensure that processes are always carried out in accord with established, written procedures.
8. Provide adequate conditions and procedures for the prevention of mixups.
9. Establish adequate procedures, with supporting documentation, for investigating and correcting failures or problems in production or quality control.
10. Provide adequate separation of quality-control responsibilities from those of production to assure independent decision making.

The pursuit of GMP is an ongoing effort which must flex with new technological developments and new understanding of existing principles. Because of the extreme importance of quality in health care of the public, the US Congress has given the responsibility of regulatory scrutiny over the manufacture and distribution of drug products to the FDA. Therefore, the operations of the pharmaceutical industry are subject to the oversight of the FDA and, with respect to manufacturing practices, to the application of the CGMPs.⁴ These regulations are discussed more fully in Chapter 110.

In concert with the pursuit of GMPs, the pharmaceutical industry has shown initiative and innovation in the extensive technological development and improvement in quality, safety and effectiveness of parenteral dosage forms in recent years. Further, outstanding innovative development in drug-delivery

systems is occurring. These factors have been additive in providing the public with outstanding parenteral dosage forms of drugs at this time in history.

General Manufacturing Process

The preparation of a parenteral product may be considered to encompass four general areas as follows:

1. Procurement and selection of the components and containers.
2. Production facilities and procedures.
3. Control of quality.
4. Packaging and labeling.

These components of the product to be procured include vehicles, solutes, containers and closures. The steps consti-

tuting production include maintaining facilities and equipment, preparing and controlling the environment, cleaning the containers and equipment, preparing the product, filtering the solution, filling containers with the product, sealing the containers and sterilizing the product. The control of quality includes the evaluation of the components, qualification of equipment, validation of processes, determination that the production has been executed within prescribed requirements and performance of necessary evaluative tests on the finished product. The final area of packaging and labeling includes all steps necessary to identify the finished product and enclose it in such manner that it is safely and properly prepared for sale and delivery to the user. The remainder of this chapter will be organized in accord with these four general areas, with emphasis on the first two areas.

Components and Containers

Establishing specifications to ensure the quality of each of the components of an injection is essential. These specifications will be coordinated with the requirements of the specific formulation and will not necessarily be identical for a particular component if used in several different formulations. For example, particle-size control may be necessary for powders used in formulating a suspension but be relatively unimportant for preparing a solution.

The most stringent chemical-purity requirements normally will be encountered with aqueous solutions, particularly if the product is to be sterilized at an elevated temperature where reaction rates will be accelerated greatly. Modification of aqueous vehicles to include a glycol, for example, usually will reduce reaction rates. Dry preparations pose relatively few reaction problems but may require definitive physical specifications for ingredients that must have certain solution or dispersion characteristics when a vehicle is added.

Containers and closures are in prolonged, intimate contact with the product and may release substances into or remove ingredients from the product. Assessment and selection of containers and closures is a necessary part of product formulation to ensure that the product retains its purity, potency and quality during the intimate contact with the container throughout its shelf-life. Administration devices that come in contact with the product should be assessed and selected with the same care as are containers and closures, even though the contact period is usually brief.

Vehicles

Since most liquid injections are quite dilute, the component present in the highest proportion is the vehicle. A vehicle normally has no therapeutic activity and is nontoxic. However, it is of great importance in the formulation since it presents to body tissues the form of the active constituent for absorption. Absorption normally occurs most rapidly and completely when a drug is presented as an aqueous solution. Modification of the vehicle with water-miscible liquids or substitution with water-immiscible liquids normally decreases the rate of absorption. Absorption from a suspension may be affected by such factors as the viscosity of the vehicle, its capacity for wetting the solid particles, the solubility equilibrium produced by the vehicle and the distribution coefficient between the vehicle and aqueous body systems.

The vehicle of greatest importance for parenteral products is water. Water of suitable quality for compounding and rinsing product contact surfaces may be prepared either by distillation or by reverse osmosis, to meet USP specifications for Water for Injection (WFI). Only by these two methods is it possible to separate adequately various liquid, gas and solid contaminating substances from water. These two methods for preparation of WFI will be discussed in this Chapter. It

should be noted that there is no unit operation more important and none more costly to install and operate than the one for the preparation of WFI.

Preparation of Water for Injection (WFI)

The source water can be expected to be contaminated with natural suspended mineral and organic substances, dissolved mineral salts, colloidal silicates and industrial chemicals. The degree of contamination will vary with the source and will be markedly different whether obtained from a well or from surface sources, such as a stream or lake. Therefore, this water normally is not of sufficient purity to prepare WFI directly. Hence, the source water usually must be pretreated by one or a combination of the following treatments: chemical softening, filtration, deionization, carbon adsorption or reverse osmosis purification. Space does not permit discussion of these processes here, but the interested reader is referred elsewhere for this information.^{5,6}

In general, a conventional still consists of a boiler (evaporator) containing feed water (distilland); a source of heat to vaporize the water in the evaporator; a headspace above the level of distilland with condensing surfaces for refluxing the vapor, thereby returning nonvolatile impurities to the distilland, a means for eliminating volatile impurities before the hot water vapor is condensed; and a condenser for removing the heat of vaporization, thereby converting the water vapor to a liquid distillate.

The specific construction features of a still and the process specifications markedly will affect the quality of distillate obtained from a still. Those required for producing high-purity water, such as WFI, must be considerably more stringent than those required for Purified Water USP. Among the factors that must be considered are:

1. The quality of the feed water will affect the quality of the distillate. Controlling the quality of the feed water is essential for meeting the required specifications for the distillate.
2. The size of the evaporator will affect the efficiency. It should be large enough to provide a low vapor velocity, thus reducing the entrainment of the distilland either as a film on vapor bubbles or as separate droplets.
3. The baffles (condensing surfaces) determine the effectiveness of refluxing. They should be designed to remove efficiently the entrainment at optimal vapor velocity, collecting and returning the heavier droplets contaminated with the distilland.
4. Redissolving volatile impurities in the distillate reduces its purity. Therefore, they should be separated efficiently from the hot water vapor and eliminated by aspirating them to the drain or venting them to the atmosphere.
5. Contamination of the vapor and distillate from the metal parts of the still can occur. Present standards for high-purity stills are that all parts contacted by the vapor or distillate should be constructed of metal coated with pure tin, 304 or 316 stainless steel or chemically resistant glass.

Conventional commercial stills designed for the production of high-purity water, such as shown in Fig 1, are available from several suppliers (*AMSCO, Barnstead, Corning, Vaponics*).

Vapor-compression stills are available in capacities from 50 to 2800 gal/hr (*Aqua-Chem, Barnstead, Meco*).

STEAM LINE

VAPOR SEPARATOR

VENT DRAPE

COMPRESSOR
DRIVEN BY EITHER ELECTRIC
MOTOR OR DIESEL ENGINE

COMPRESSED STEAM
APPROX T = 224°F

OVERFLOW

FEED

CONDENSATE

DOUBLE PIPE HEAT EXCHANGER

NOTE: SUPPLEMENTAL HEAT
ELECTRIC UNIT-ELECTRIC HEATER
DIESEL UNIT-ENGINE COOLING &
EXHAUST SYSTEMS

VENT
OVERFLOW APPROX. T = 80°F
FEED APPROX T = 80°F
CONDENSATE APPROX T = 74°F

the steam is at atmospheric pressure and must be condensed in a heat exchanger.

Reverse Osmosis (RO)—As the name suggests, the natural process of selective permeation of molecules through a semipermeable membrane separating two aqueous solutions of different concentrations is reversed. Pressure, usually between 200 and 400 psig, is applied to overcome osmotic pressure and force pure water to permeate through the membrane. Membranes, usually composed of cellulose esters or polyamides, are selected to provide an efficient rejection of contaminant molecules in raw water. The molecules most difficult to remove are small inorganic ones such as sodium chloride. Passage through two membranes in series is sometimes used to increase the efficiency of removal of these small molecules and to decrease the risk of structural failure of a membrane to remove other contaminants, such as bacteria and pyrogens. For additional information, see *Reverse Osmosis* in Chapter 37 and *Water* in Chapters 80 and 86.

Fig 3. Multiple-effect still. (courtesy, Dekker); taken from Avis KE, Lieberman HA, Lachman L; *Pharmaceutical Dosage Forms: Parenteral Medications*, vol 2, 2nd ed, Dekker, New York, 1993.

Whichever system is used for the preparation of WFI, validation is required to be sure that the system consistently and reliably will produce the chemical, physical and microbiological quality of water required. Such validation should start with the determined characteristics of the source water and include the pretreatment, production, storage and distribution systems. All of these systems together determine the ultimate quality of the WFI. Because of space limitations here, more details concerning the design, operation and validation of these highly important systems may be found in other literature sources.^{5,6}

Storage and Distribution—The rate of production of WFI usually is not sufficient to meet processing demands; therefore, it is collected in a holding tank for subsequent use. In large operations the holding tanks may have a capacity of several thousand gallons and be a part of a continuously operating system. In such instances the USP requires that the WFI be held at a temperature too high for microbial growth to occur. Normally, this temperature is a constant 80°.

The USP also permits the WFI to be stored at room temperature, but for a maximum of 24 hours. Under such conditions the WFI usually is collected as a batch for a particular use with any unused water being discarded within 24 hours. Such a system requires frequent sanitization to minimize the risk of viable microorganisms being present. The stainless-steel storage tanks in such systems usually are connected to a welded stainless-steel distribution loop supplying the various use sites with a continuously circulating water supply. The tank is provided with a hydrophobic membrane vent filter capable of excluding bacteria and nonviable particulate matter. Such a vent filter is necessary to permit changes in pressure during filling and emptying. The construction material for the tank and connecting lines is usually electropolished 316L stainless steel with welded pipe. The tanks also may be lined with glass or a coating of pure tin. Such systems are very carefully designed and constructed and often constitute the most costly installation within the plant.

When the water cannot be used at 80°, heat exchangers must be installed to reduce the temperature at the point of use. Bacterial retentive filters should not be installed in such systems because of the risk of bacterial buildup on the filters and the consequential release of pyrogenic substances.

Purity—The USP monographs provide standards of purity for WFI and Sterile Water for Injection (SWFI). A few of these standards require comment.

SWFI must meet the requirements of the USP Sterility Test, but WFI need not since it is to be used in a product which will be sterilized. Both must contain not more than 0.25 Endotoxin units per mL.

The limits for total solids varies in the two monographs. The larger the surface area of the glass container per unit volume of water, the greater the amount of glass constituents that may be leached into the water, particularly during the elevated temperature of steam sterilization. Therefore, the latter treatment will affect the total solids content of SWFI.

The WFI monograph stipulates a maximum of 10 ppm of total solids but SWFI may contain 20 to 40 ppm. This is generally considered to be much too high to assure a quality of water that permits the stable formulation of many drugs. A relatively few metallic ions present often can render a formulation unstable. Therefore, it is common practice to set a limit of 0.1 ppm or less of ionic contaminants expressed as sodium chloride.

Ionic contaminant level is not the same as total solids; the former is a measure of only the ionic content, while the latter is a measure of the undissociated constituents as well. The ionic content of water can be measured very easily by means of a conductivity meter which frequently is used as an indicator of the purity. The results are expressed in one of three terms: as sodium chloride ions, as resistance in ohms or megohms or as conductance in micromhos (or microSiemens). Ohms and mhos have a reciprocal relationship to each other, but they are related to ppm sodium chloride by an experimentally determined curve. The USP is considering introducing

a conductivity requirement. To give one point of comparison, 0.1 ppm sodium chloride is equal to approximately 1.01 megohms and 0.99 micromhos. It should be mentioned that conductivity measurements give no direct indication of pyrogen content since pyrogens are undissociated organic compounds.

WFI and SWFI may not contain added substances. Bacteriostatic Water for Injection (BWFI) may contain one or more suitable antimicrobial agents in containers of 30 mL or less. This restriction is designed to prevent the administration of a large quantity of a bacteriostatic agent that probably would be toxic in the accumulated amount of a large volume of solution, even though the concentration was low.

The USP also provides monographs giving the specifications for Sterile Water for Inhalation and Sterile Water for Irrigation. The USP should be consulted for the minor differences between these specifications and those for SWFI.

Types of Vehicles

Aqueous Vehicles—Certain aqueous vehicles are recognized officially because of their valid use in parenterals. Often they are used as isotonic vehicles to which a drug may be added at the time of administration. The additional osmotic effect of the drug may not be enough to produce any discomfort when administered. These vehicles include Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection and Lactated Ringer's Injection.

Water-Miscible Vehicles—A number of solvents that are miscible with water have been used as a portion of the vehicle in the formulation of parenterals. These solvents are used primarily to affect the solubility of certain drugs and to reduce hydrolysis. The most important solvents in this group are ethyl alcohol, polyethylene glycol and propylene glycol. Ethyl alcohol is used particularly in the preparation of solutions of cardiac glycosides and the glycols in solutions of barbiturates, certain alkaloids and certain antibiotics. Such preparations usually are given intramuscularly.

These solvents, as well as nonaqueous vehicles, have been reviewed by Spiegel and Noseworthy.⁷

Nonaqueous Vehicles—The most important group of nonaqueous vehicles are the fixed oils. The USP provides specifications for such vehicles, indicating that the fixed oils must be of vegetable origin so that they will be metabolized, will be liquid at room temperature and will not become rancid readily. The USP also specifies limits for the degree of unsaturation and free fatty acid content. The oils most commonly used are corn oil, cottonseed oil, peanut oil and sesame oil. Fixed oils are used particularly as vehicles for certain hormone preparations. The label must state the name of the vehicle so that the user may beware in case of known sensitivity or other reactions to it.

Solutes

Bulk pharmaceutical chemicals should be prepared under conditions designed to preclude the introduction of chemical, physical and microbiological contamination. To the extent possible, the system of manufacture should be a closed system, particularly if the chemical is to be used in a parenteral formulation. Further, the contact surfaces of all process equipment should be inert to the chemicals being processed. Where the chemicals are to be used in relatively small amounts, marketed commercial grades may be the only forms available. In such instances, the best chemical grade obtainable should be used. It should be obvious that if a few ppm of ionic contaminants in WFI may cause stability problems, a similar level of contamination in the solute itself may, likewise, cause stability problems. Metallic catalysis of chemical reactions is one which is encountered frequently.

Other factors to be considered with respect to the quality of solutes include the level of microbial and pyrogenic contami-

nat
cal
dirt
A
sub
qua
E:
zoat
Ph
isou
E:
gase
Ph
"pri
veni
ited
reta
prej
V
froi
visi
oxi
wis
sub
wit
toti
A
bia
be
cor
att
isr
dra
anc
Pri
bia
of
cro
pre
mc
F
E
I
C
mc
Me
zo:
us
ria
ev
ylr
fel
ter
pa
tic
is
tiv
be
th
ex
be
th
ru
ba
by
nc
pr
st
bi
in

nation, solubility characteristics as determined by the chemical or physical form of the compound and freedom from gross dirt.

Added Substances—The USP includes in this category all substances added to a preparation to improve or safeguard its quality. An added substance may:

Effect solubility, as does sodium benzoate in Caffeine and Sodium Benzoate Injection.

Provide patient comfort, as do substances added to make a solution isotonic.

Enhance the chemical stability of a solution, as do antioxidants, inert gases, chelating agents and buffers.

Protect a preparation against the growth of microorganisms. The term "preservative" sometimes is applied only to those substances which prevent the growth of microorganisms in a preparation. However, such limited use is inappropriate, being better used for all substances that act to retard or prevent the chemical, physical or biological degradation of a preparation.

While added substances may prevent a certain reaction from taking place, they may induce others. Not only may visible incompatibilities occur, but hydrolysis, complexation, oxidation and invisible reactions may decompose or otherwise inactivate the therapeutic agent or other added substances. Therefore, added substances must be selected with due consideration and investigation of their effect on the total formulation.

Antimicrobial Agents—The USP states that antimicrobial agents in bacteriostatic or fungistatic concentrations must be added to preparations contained in multiple-dose containers. They must be present in adequate concentration at the time of use to prevent the multiplication of microorganisms inadvertently introduced into the preparation while withdrawing a portion of the contents with a hypodermic needle and syringe. The USP provides a test for *Antimicrobial Preservative Effectiveness* to determine that an antimicrobial substance or combination adequately inhibits the growth of microorganisms in a parenteral product. Because antimicrobials may have inherent toxicity for the patient, the USP prescribes concentration limits for those that are used commonly in parenteral products, as follows:

Phenylmercuric nitrate and thimerosal 0.01%.
Benzethonium chloride and benzalkonium chloride 0.01%.
Phenol or cresol 0.5%.
Chlorobutanol 0.5%.

The above limit is rarely used for phenylmercuric nitrate, most frequently being employed in a concentration of 0.002%. Methyl *p*-hydroxybenzoate 0.18% and propyl *p*-hydroxybenzoate 0.02% in combination, and benzyl alcohol 2% also are used frequently. In oleaginous preparations, no antibacterial agent commonly employed appears to be effective. However, it has been reported that hexylresorcinol 0.5% and phenylmercuric benzoate 0.1% are moderately bactericidal. A few therapeutic compounds have been shown to have antibacterial activity, thus obviating the need for added agents.

Antimicrobial agents must be studied with respect to compatibility with all other components of the formula. In addition, their activity must be evaluated in the total formula. It is not uncommon to find that a particular agent will be effective in one formulation but ineffective in another. This may be due to the effect of various components of the formula on the biological activity or availability of the compound; for example, the binding and inactivation of esters of *p*-hydroxybenzoic acid by macromolecules such as Polysorbate 80 or the reduction of phenylmercuric nitrate by sulfide residues in rubber closures. A physical reaction encountered is that bacteriostatic agents sometimes are removed from solution by rubber closures.

Single-dose containers and pharmacy bulk packs which do not contain antimicrobial agents are expected to be used promptly after opening or to be discarded. Large-volume single-dose containers may not contain an added antimicrobial preservative. Therefore, special care must be exercised in storing such products after the containers have been opened

to prepare an admixture, particularly those that can support the growth of microorganisms, such as total parenteral nutrition (TPN) solutions. It should be noted that while refrigeration slows the growth of most microorganisms, it does not prevent their growth.

Buffers are used primarily to stabilize a solution against the chemical degradation that might occur if the pH changes appreciably. Buffer systems employed should normally have as low a buffer capacity as feasible in order not to disturb significantly the body buffer systems when injected. In addition, the buffer range and effect on the activity of the product must be evaluated carefully. The acid salts most frequently employed as buffers are citrates, acetates and phosphates.

Antioxidants are required frequently to preserve products because of the ease with which many drugs are oxidized. Sodium bisulfite 0.1% is used most frequently. The use of sulfites has been reviewed by Schroeter.⁸ Acetone sodium bisulfite, sodium formaldehyde sulfoxylate and thiourea also are used sometimes. The sodium salt of ethylenediaminetetraacetic acid has been found to enhance the activity of antioxidants in some cases, apparently by chelating metallic ions that would otherwise catalyze the oxidation reaction.

Displacing the air (oxygen) in and above the solution by purging with an inert gas, such as nitrogen, also can be used as a means to control oxidation of a sensitive drug. Process control is required to be assured that every container is deaerated adequately and uniformly.

Tonicity Agents are used in many parenteral and ophthalmic products to control the tonicity. However, not all preparations need to be isotonic. The agents most commonly used are electrolytes and mono- or disaccharides. This subject is considered much more extensively in Chapter 36.

Pyrogens (Endotoxins)

Pyrogens are products of metabolism of microorganisms. The most potent pyrogenic substances (endotoxins) are constituents of the cell wall of Gram-negative bacteria. Gram-positive bacteria and fungi also produce pyrogens but of lower potency and of different chemical nature. Endotoxins are high-molecular-weight (about 20,000 daltons) lipopolysaccharides. Studies have shown that the lipid portion of the molecule is responsible for the biological activity. Since endotoxins are the most potent pyrogens and Gram-negative bacteria are ubiquitous in the environment, this discussion will focus on endotoxins and the risk of their presence as contaminants in sterile products.

Pyrogens, when present in parenteral drug products and injected into patients, can cause fever, chills, pain in the back and legs, and malaise. While pyrogenic reactions are rarely fatal, they can cause serious discomfort and, in the seriously ill patient, shock-like symptoms that can be fatal. The intensity of the pyrogenic response and its degree of hazard will be affected by the medical condition of the patient, the potency of the pyrogen, the amount of the pyrogen and the route of administration (intrathecal is most hazardous followed by intravenous, intramuscular and subcutaneous). When bacterial (exogenous) pyrogens are introduced into the body, leucocytic phagocytosis is believed to occur and endogenous pyrogen is produced. The endogenous pyrogen then produces the familiar physiological effects. Space will not permit further elaboration of these matters here, but the reader is referred to the work by Pearson⁹ if more information is needed.

Control of Pyrogens—Pyrogens are contaminants if present in parenteral drug products, and should not be there. In general, it is impractical, if not impossible, to remove pyrogens once present without adversely affecting the drug product. Therefore, the emphasis should be on the prevention of the introduction or development of pyrogens in all aspects of the compounding and processing of the product.

Pyrogens may enter a preparation through any means that will introduce living or dead microorganisms. However, current technology generally permits the control of such contamination, and the presence of pyrogens in a finished product is

indicative of processing under inadequately controlled clean conditions. It also should be noted that time for microbial growth to occur increases the risk for elevated levels of pyrogens. Therefore, compounding and manufacturing processes should be carried out as expeditiously as possible, preferably planning completion of the process, including sterilization, within one work day.

Pyrogens can be destroyed by heating at high temperatures. The recommended procedure for depyrogenation of glassware and equipment is heating at a temperature of 250° for 45 minutes. It has been reported that 650° for 1 minute or 180° for 4 hours likewise will destroy pyrogens. The usual autoclaving cycle will not do so. Heating with strong alkali or oxidizing solutions will destroy pyrogens. It has been claimed that thorough washing with detergent will render glassware pyrogen-free if subsequently rinsed thoroughly with pyrogen-free water. Plastic containers and devices must be protected from pyrogenic contamination during manufacture and storage since known ways to destroy pyrogens will affect the plastic adversely. It has been reported that anion-exchange resins and positively charged membrane filters will remove pyrogens from water. Also, reverse osmosis will eliminate them. However, the most reliable method for their elimination from water is distillation.

A method that has been used for the removal of pyrogens from solutions is adsorption on adsorptive agents. However, since the adsorption phenomenon also may cause selective removal of chemical substances from the solution, this method has limited application. Other in-process methods for their destruction or elimination include selective extraction procedures and careful heating with dilute alkali, dilute acid or mild oxidizing agents. In each instance, the method must be studied thoroughly to be sure it will not have an adverse effect on the constituents of the product. Developments in ultrafiltration now make possible pyrogen separation on a molecular weight basis and the process of tangential flow is making large-scale processing a reality.

Sources of Pyrogens—Through understanding the means by which pyrogens may contaminate parenteral products, their control becomes more achievable. Therefore, it is important to know that water is probably the greatest potential source of pyrogenic contamination, since water is essential for the growth of microorganisms. When microorganisms metabolize, pyrogens will be produced. Therefore, raw water can be expected to be pyrogenic and only when it is appropriately treated to render it free from pyrogens, such as WFI, should it be used for compounding the product or rinsing product contact surfaces such as tubing, mixing vessels and rubber closures. Even when such rinsed equipment and sup-

plies are left wet and improperly exposed to the environment, there is a high risk that they will become pyrogenic. As stated previously, although proper distillation will provide pyrogen-free water, storage conditions must be such that microorganisms are not introduced and subsequent growth is prevented.

Other potential sources of contamination are containers and equipment. Pyrogenic materials adhere strongly to glass and other surfaces. Residues of solutions in used equipment often become bacterial cultures with subsequent pyrogenic contamination. Since drying does not destroy pyrogens, they may remain in equipment for long periods. Adequate washing will reduce and subsequent dry-heat treatment can render contaminated equipment suitable for use. However, all such processes must be validated to assure their effectiveness.

Solutes may be a source of pyrogens. For example, the manufacturing of bulk chemicals may involve the use of pyrogenic water for process steps such as crystallization, precipitation or washing. Bulk drug substances derived from fermentation will almost certainly be heavily pyrogenic. Therefore, all lots of solutes used to prepare parenteral products should be tested to ensure that they will not contribute unacceptable quantities of endotoxin to the finished product.

The manufacturing process must be carried out with great care and as rapidly as possible to minimize the risk of microbial contamination. Preferably, no more product should be prepared than can be processed, completely within one working day, including sterilization.

Containers

Containers are an integral part of the formulation of an injection. No container is totally insoluble or does not in some way affect the liquid it contains, particularly if the liquid is aqueous. Therefore, the selection of a container for a particular injection must be based on a consideration of the composition of the container, as well as of the solution, and the treatment to which it will be subjected.

Table 1 provides a generalized comparison of the three compatibility properties—leaching, permeation and adsorption—of container materials most likely to be involved in the formulation of aqueous parenterals. Further, the integrity of the container/closure system depends upon several characteristics, including container opening finish, closure modulus, durometer and compression set, and aluminum seal application force. These considerations have been reviewed by Morton.¹⁰

Table 1—Comparative Compatibility Properties of Container Materials

	Leaching		Permeation		Adsorption (selective) extent ^a
	Extent ^a	Potential leachables	Extent ^a	Potential agents	
Glass					
Borosilicate	1	Alkaline earth and heavy metal oxides	0	N/A	2
Soda-lime	5	Alkaline earth and heavy metal oxides	0	N/A	2
Plastic polymers					
Polyethylene					
Low density	2	Plasticizers, antioxidants	5	Gases, water vapor, other molecules	2
High density	1	Antioxidants	3	Gases, water vapor, other molecules	2
PVC	4	HCl, especially plasticizers, antioxidants, other stabilizers	5	Gases, especially water vapor and other molecules	2
Polyolefins	2	Antioxidants	2	Gases, water vapor, other molecules	2
Polypropylene	2	Antioxidants, lubricants	4	Gases, water vapor	1
Rubber polymers					
Natural and related synthetic	5	Heavy metal salts, lubricants, reducing agents	3	Gases, water vapor	3
Butyl	3	Heavy metal salts, lubricants, reducing agents	1	Gases, water vapor	2
Silicone	2	Minimal	5	Gases, water vapor	1

^a Approximate scale of 1 to 5 with "1" as the lowest.

Plastic

Thermoplastic polymers have been established as packaging materials for sterile preparations such as large-volume parenterals, ophthalmic solutions and, increasingly, for small-volume parenterals. For such use to be acceptable a thorough understanding of the characteristics, potential problems and advantages for use must be developed. A historical review of these factors relative to pharmaceuticals has been prepared by Autian.¹¹ A recent discussion of polymers for IV solutions has been published by Lambert.¹² Autian stated that three principal problem areas exist in using these materials; namely,

1. Permeation of vapors and other molecules in either direction through the wall of the plastic container.
2. Leaching of constituents from the plastic into the product.
3. Sorption (absorption and/or adsorption) of drug molecules or ions on the plastic material.

Permeation, the most extensive problem, may be troublesome by permitting volatile constituents, water or selected drug molecules to migrate through the wall of the container to the outside and thereby be lost. This problem has been resolved, for example, by the use of an overwrap in the packaging of IV solutions in PVC bags to prevent the loss of water during storage. Reverse permeation also may occur by which oxygen or other molecules may penetrate to the inside of the container and cause oxidative or other degradation of susceptible constituents. Leaching may be a problem when certain constituents in the plastic formulation, such as plasticizers or antioxidants, migrate into the product. Thus, plastic polymer formulations should have as few additives as possible, an objective characteristically achievable for most plastics being used for parenteral packaging. *Sorption* is a problem on a selective basis, that is, sorption of a few drug molecules occurs on select polymers. For example, sorption of insulin, vitamin A acetate and warfarin sodium has been shown to occur on PVC bags and tubing when these drugs were present as additives in IV admixtures. A brief summary of some of these compatibility relationships is given in Table 1.

One of the principle advantages of using plastic packaging materials is that they are not breakable as is glass; also, there is a substantial weight reduction. The flexibility of the low-density polyethylene polymer, for ophthalmic preparations, makes it possible to squeeze the side wall of the container and discharge one or more drops without introducing contamination into the remainder of the product. The flexible bags of polyvinyl chloride or select polyolefins, currently in use for large-volume intravenous fluids, have the added advantage that no air interchange is required; the flexible wall simply collapses as the solution flows out of the bag.

Most plastic materials have the disadvantage that they are not as clear as glass and, therefore, inspection of the contents is impeded. In addition, many of these materials will soften or melt under the conditions of thermal sterilization. However, careful selection of the plastic used and control of the autoclave cycle has made thermal sterilization of some products possible, large-volume parenterals in particular. Ethylene oxide or radiation sterilization may be employed for the empty container with subsequent aseptic filling. However, careful evaluation of the residues from ethylene oxide or its degradation products and their potential toxic effect must be undertaken. Investigation is required concerning potential interactions and other problems that may be encountered when a parenteral product is packaged in plastic. For further details see Chapter 85.

Glass

Glass is employed as the container material of choice for most SVPs. It is composed principally of silicon dioxide with varying amounts of other oxides such as sodium, potassium, calcium, magnesium, aluminum, boron and iron. The basic structural network of glass is formed by the silicon oxide

tetrahedron. Boric oxide will enter into this structure, but most of the other oxides do not. The latter are only loosely bound, are present in the network interstices and are relatively free to migrate. These migratory oxides may be leached into a solution in contact with the glass, particularly during the increased reactivity of thermal sterilization. The oxides thus dissolved may hydrolyze to raise the pH of the solution, catalyze reactions or enter into reactions. Additionally, some glass compounds will be attacked by solutions and, in time, dislodge glass flakes into the solution. Such occurrences can be minimized by the proper selection of the glass composition.¹³

Types—The USP has aided in this selection by providing a classification of glass; namely,

Type I, a borosilicate glass.

Type II, a soda-lime treated glass.

Type III, a soda-lime glass.

NP, a soda-lime glass not suitable for containers for parenterals.

Type I glass is composed principally of silicon dioxide and boric oxide, with low levels of the non-network-forming oxides. It is a chemically resistant glass (low leachability) also having a low thermal coefficient of expansion.

Types II and III glass compounds are composed of relatively high proportions of sodium oxide and calcium oxide. This makes the glass chemically less resistant. Both types melt at a lower temperature, are easier to mold into various shapes and have a higher thermal coefficient of expansion than Type I. While there is no one standard formulation for glass among manufacturers of these USP type categories, Type II glass usually has a lower concentration of the migratory oxides than Type III. In addition, Type II has been treated under controlled temperature and humidity conditions with sulfur dioxide to dealkalize the interior surface of the container. While it remains intact, this surface will increase substantially the chemical resistance of the glass. However, repeated exposures to sterilization and alkaline detergents will break down this dealkalized surface and expose the underlying soda-lime compound.

The glass types are determined from the results of two USP tests: the Powdered Glass Test and the Water Attack Test. The latter is used only for Type II glass and is performed on the whole container, because of the dealkalized surface; the former is performed on powdered glass, which exposes internal surfaces of the glass compound. The results are based upon the amount of alkali titrated by 0.02 N sulfuric acid after an autoclaving cycle with the glass sample in contact with a high-purity distilled water. Thus, the *Powdered Glass Test* challenges the leaching potential of the interior structure of the glass while the *Water Attack Test* challenges only the intact surface of the container.

Selecting the appropriate glass composition is a critical facet of determining the overall specifications for each parenteral formulation.

In general, Type I glass will be suitable for all products, although sulfur dioxide treatment sometimes is used for a further increase in resistance. Because cost must be considered, one of the other less expensive types may be acceptable. Type II glass may be suitable, for example, for a solution which is buffered, has a pH below 7 or is not reactive with the glass. Type III glass usually will be suitable principally for anhydrous liquids or dry substances. However, some manufacturer-to-manufacturer variation in glass composition should be anticipated within each glass type. Therefore, for highly chemically sensitive parenteral formulations it may be necessary to specify both USP Type and a specific manufacturer.

Physical Characteristics—Some of the physical shapes of glass ampuls and vials are illustrated in Fig 3. Commercially available containers vary in size from 0.5 to 1000 mL. Sizes up to 100 mL may be obtained as ampuls and vials, and larger sizes as bottles. The latter are used mostly for intravenous and irrigating solutions. Smaller sizes are also available as cartridges. Ampuls and cartridges are drawn from glass tubing. The smaller vials may be made by mold-

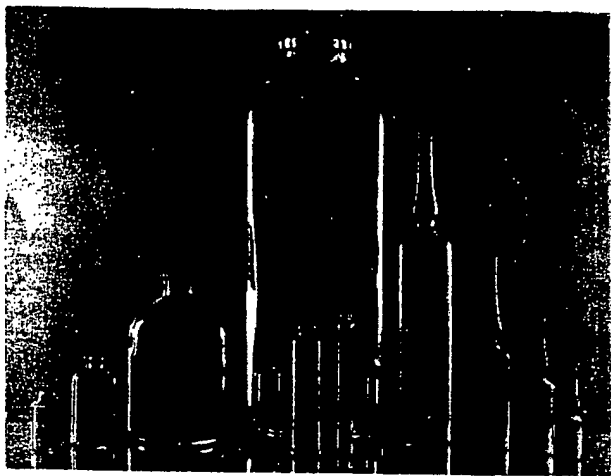


Fig 4. Various types of ampuls and multiple-dose vials for parenterals (courtesy, Kimble).

ing or from tubing. Larger vials and bottles are made only by molding. Containers made by drawing tubing are generally optically clearer and have a thinner wall than molded containers (see Fig 4). Molded containers are uniform in external dimensions, stronger and heavier.

Easy-opening ampuls that permit the user to break off the tip at the neck constriction without the use of a file are weakened at the neck by scoring or applying a ceramic paint having a different coefficient of thermal expansion. An example of a modification of container design to meet a particular need is the double-chambered vial, under the name Univial (*Univial*), designed to contain a freeze-dried product in the lower and solvent in the upper chamber. Other examples are wide-mouth ampuls with flat or rounded bottoms to facilitate filling with dry materials or suspensions, and various modifications of the cartridge for use with disposable dosage units.

Glass containers must be strong enough to withstand the physical shocks of handling and shipping and the pressure differentials that develop, particularly during the autoclave sterilization cycle. They must be able to withstand the thermal shock resulting from large temperature changes during processing, for example, when the hot bottle and contents are exposed to room air at the end of the sterilization cycle. Therefore, a glass having a low coefficient of thermal expansion is necessary. The container also must be transparent to permit inspection of the contents.

Preparations which are light-sensitive must be protected by placing them in amber glass containers or by enclosing flint glass containers in opaque cartons labeled to remain on the container during the period of use. It should be noted that the amber color of the glass is imparted by the incorporation of potentially leachable heavy metals, mostly iron and manganese, which may act as catalysts for oxidative degradation reactions. Silicone coatings sometimes are applied to containers to produce a hydrophobic surface, for example, as a means of reducing the friction of a rubber-tip of a syringe plunger.

The size of single-dose containers is limited to 1000 mL by the USP and multiple-dose containers to 30 mL, unless stated otherwise in a particular monograph. Multiple-dose vials are limited in size to reduce the number of punctures for withdrawing doses and the accompanying risk of contamination of the contents. As the name implies, single-dose containers are opened with aseptic care and the contents used at one time. These may range in size from 1000-mL bottles to 1-mL or less ampuls, vials or syringes. The integrity of the container is destroyed when opened so that the container cannot be closed again.

A multiple-dose container is designed so that more than one dose can be withdrawn at different times, the container maintaining a seal between uses. It should be evident that with



Fig 5. Extended view of scaling components for a multiple-dose vial (courtesy, West).

full aseptic precautions, including sterile syringe and needle for withdrawing the dose and disinfection of the exposed surface of the closure, there is still a substantial risk of introducing contaminating microorganisms and viruses into the contents of the vial. Because of this risk, the USP requires that all multiple-dose vials must contain an antimicrobial agent or be inherently antimicrobial, as determined by the USP *Antimicrobial Preservatives-Effectiveness* tests. There are no comparable antiviral effectiveness tests, nor are antiviral agents available for such use. In spite of the advantageous flexibility of dosage provided by multiple-dose vials, single-dose, disposable container units provide the clear advantage of greater sterility assurance and patient safety.

Rubber Closures

In order to permit introduction of a needle from a hypodermic syringe into a multiple-dose vial and provide for resealing as soon as the needle is withdrawn, each vial is sealed with a rubber closure held in place by an aluminum cap. Figure 5 illustrates how this is done. This principle also is followed for single-dose containers of the cartridge type, except that there is only a single introduction of the needle to make possible the withdrawal or expulsion of the contents.

Rubber closures are composed of multiple ingredients that are plasticized and mixed together at an elevated temperature on milling machines. Subsequently, the plasticized mixture is placed in molds and vulcanized (cured) under high temperature and pressure. During vulcanization the polymer strands are cross-linked by the vulcanizing agent, assisted by the accelerator and activator, so that motion is restricted and the molded closure acquires the elastic, resilient character required for its use. Ingredients not involved in the cross-linking reactions remain dispersed within the compound and,

Table 2—Examples of Ingredients in Rubber Closures

Ingredient	Examples
Elastomer	Natural rubber (latex) Butyl rubber Neoprene
Vulcanizing (curing) agent	Sulfur Peroxides
Accelerator	Zinc dibutyldithiocarbamate
Activator	Zinc oxide Stearic acid
Antioxidant	Dilauryl thiodipropionate
Plasticizer/lubricant	Paraffinic oil Silicone oil
Fillers	Carbon black Clay
Pigments	Barium sulfate Inorganic oxides Carbon black

along
finish
giver
Th
a par
to fra
ity is
or ot
derr
vide
need
piec
is pu
to so
tion
perm
eval
the U
Th
pour
the c
in Ta

A
may
it is
dure
facil
mus
stan
the

Arr

Th
func
the
pac
stru
trol
requ
env
to th
bull
con
mic
cou
per
on
dire
nar
Alth
tha
mu
crit
of f
airl
cur
the
crit
pas
sis
tur
sh
thr
ase
)
pri
eit

along with the degree of curing, affect the properties of the finished closure. Examples of rubber-closure ingredients are given in Table 2.

The physical properties to be considered in the selection of a particular formulation include elasticity, hardness, tendency to fragment and permeability to vapor transfer. The elasticity is critical in establishing a seal with the lip and neck of a vial or other opening, and in resealing after withdrawal of a hypodermic needle from a vial closure. The hardness should provide firmness by not excessive resistance to the insertion of a needle through the closure, while minimal fragmentation of pieces of rubber should occur as the hollow shaft of the needle is pushed through the closure. While vapor transfer occurs to some degree with all rubber formulations, appropriate selection of ingredients makes it possible to control the degree of permeability. Physicochemical and toxicological tests for evaluating rubber closures are described in section (381) in the USP.

The ingredients dispersed throughout the rubber compound may be subject to leaching into the product contacting the closure. These ingredients, examples of which are given in Table 2, pose potential compatibility interactions with prod-

uct ingredients if leached into the product solution, and these effects must be evaluated.^{14,15} Further, some ingredients must be evaluated for potential toxicity. In order to reduce the problem of leachables, attempts have been made to coat the product contact surfaces of closures with various polymers, the most successful being Teflon, or to treat the closures in ways considered trade secrets.

The physical shape of some typical closures may be seen in Fig 5. Most of them have a lip and a protruding flange that extends into the neck of the vial or bottle. Many disk closures are being used now, particularly in the high-speed packaging of antibiotics. Slotted closures are used on freeze-dried products to make it possible to insert the closure part way into the neck of the vial during the drying phase of the cycle. Partial insertion provides limited protection from contamination while permitting water vapor to escape from the drying product. The plunger type is used to seal one end of a cartridge. At the time of use, the plunger expels the product by a needle inserted through the closure at the distal end of the cartridge. Intravenous solution closures often have permanent holes for adapters of administration sets; irrigating solution closures usually are designed for pouring.

Production Facilities

A product having components of the best quality quickly may become totally unacceptable if the environment in which it is processed is contaminated or if the manufacturing procedure is not carried out properly. Therefore, the production facilities and the procedure used in processing the product must meet standards adequate for the task. The nearer these standards approach perfection, the better and safer should be the product.

Arrangement of Area

The production area can be considered in terms of five functional areas: the cleanup area, the compounding area, the aseptic area, the quarantine area and the finishing or packaging area. All of these should be designed and constructed for cleaning ease, appropriate environmental control, efficient operation and personnel comfort. The extra requirements for the aseptic area are designed to provide an environment where, for example, an injection may be exposed to the environment for a brief period during subdivision from a bulk container to the individual-dose containers without becoming contaminated. Contaminants such as dust, lint and microorganisms normally are found floating in the air, lying on counters and other surfaces, on clothing and body surfaces of personnel, in the exhaled breath of personnel and deposited on the floor. The design and control of an aseptic area is directed toward so reducing the presence of these contaminants that they are no longer a hazard to aseptic filling. Although the aseptic area must be adjacent to support areas so that an efficient flow of components may be achieved, barriers must be provided to minimize ingress of contaminants to the critical aseptic area. Such barriers may consist of a variety of forms, including sealed walls, manual or automatic doors, airlock pass-throughs, ports of various types or plastic curtains. Figure 6 shows an example of a floor plan in which the two fill rooms and the staging area constitute the walled critical aseptic area, access to which is only by means of pass-through airlocks. Adjacent support areas (rooms) consist of glass preparation, equipment wash, capping, manufacturing (compounding) and various storage areas. Figure 7 shows an adjacent arrangement with the utilization of a through-the-wall port for passage of a filtrate into the critical aseptic filling room.

Flow Plan—In general, the components for a parenteral product flow from the stockroom for released components, either to the compounding area, as for ingredients of the

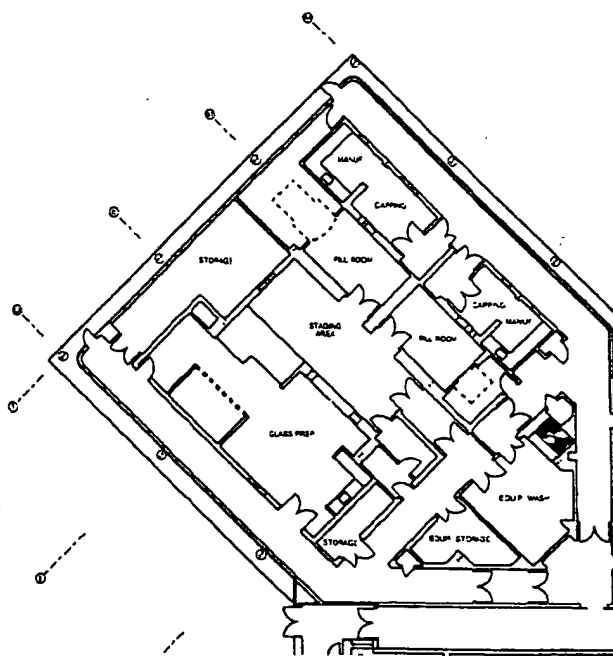


Fig 6. Floor plan of aseptic filling rooms and staging room with adjacent support areas (courtesy, Glaxo).

formula, or to the cleanup area, as for containers and equipment. See Fig 8 for a process-flow diagram. After proper processing in these areas, the components flow into the security of the aseptic area for filling of the product in appropriate containers. From there the product passes into the quarantine area where it is held until all necessary tests have been performed. If the product is to be sterilized in its final container, its passage normally is interrupted after leaving the aseptic area for subjection to the sterilization process. After the results from all tests are known and the product has been found to comply with its release specifications, it passes to the finishing area for final labeling and packaging. There sometimes are variations from this flow plan to meet the specific needs of an individual product or to conform to existing facilities. Automated operations convey the components



Fig 7. Product filtration from the aseptic staging room through a port into the aseptic filling room (courtesy, The University of Tennessee College of Pharmacy).

from one area to another with little or no handling by operators.

Cleanup Area—The cleanup area is constructed to withstand moisture, steam and detergents. The ceiling, walls and floor should be constructed of impervious materials so that moisture will run off and not be held. One of the "spray-on-tile" finishes with a vinyl or epoxy sealing coat provides a continuous surface free from all holes or crevices. All such surfaces can be washed at regular intervals to keep them thoroughly clean. These areas should be exhausted adequately so that the heat and humidity will be removed for the comfort of personnel. Precautions must be taken to prevent the accumulation of dirt and the growth of microorganisms, especially in the presence of high humidity and heat. In this area preparation for the filling operation, such as assembling equipment, is undertaken. Adequate sink and counter space must be provided. While this area does not need to be aseptic, it must be cleanable and kept clean and the microbial load must be monitored and controlled. Precautions also must be taken to prevent deposit of particles or other contaminants on clean containers and equipment.

Compounding Area—In this area the formula is compounded. Although it is not essential that this area be aseptic, control of microorganisms and particulates should be more stringent than in the cleanup area. For example, means may need to be provided to control dust generated from weighing and compounding operations. Cabinets and counters should, preferably, be constructed of stainless steel. They should fit snugly to walls and other furniture so that there are no catch areas for dirt to accumulate. The ceiling, walls and floor should be constructed similar to those for the cleanup area.

Aseptic Area

This area requires construction features designed for maximum microbial and particulate control. The ceiling, walls

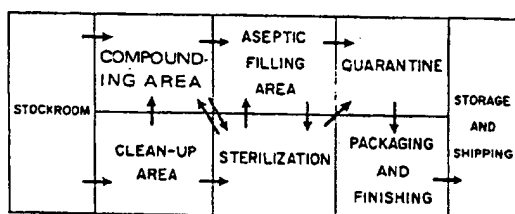


Fig 8. Process-flow diagram.

and floor must be sealed so that they may be washed and sanitized with a disinfectant, as needed. All counters should be constructed of stainless steel and hung from the wall so that there are no legs to accumulate dirt where they rest on the floor. All light fixtures, utility service lines and ventilation fixtures should be recessed in the walls or ceiling to eliminate ledges, joints and other locations for the accumulation of dust and dirt. As much as possible, tanks containing the compounded product should remain outside the aseptic filling area and the product fed into the area through hose lines. Figure 7 shows such an arrangement. Proper sanitization is required if the tanks must be moved in. Mechanical equipment that is located in the aseptic area should be housed as completely as possible within a stainless-steel cabinet in order to seal the operating parts and their dirt-producing tendencies from the aseptic environment. Mechanical parts that will contact the parenteral product should be demountable so that they can be sterilized.

Personnel entering the aseptic area should enter only through an airlock. They should be attired in sterile coveralls with sterile hats, masks, goggles and foot covers. Movement within the room should be minimal and in-and-out movement rigidly restricted during a filling procedure. The requirements for room preparation and the personnel may be relaxed somewhat if the product is to be sterilized terminally in a sealed container. Some are convinced, however, that it is better to have one standard procedure meeting the most rigid requirements.

Air Cleaning

The air in these areas can be one of the greatest sources of contamination. It need not be, however, because several methods are available for providing clean air that is essentially free from dirt particles and microorganisms.

To provide such air, it must be cleaned thoroughly of all contaminants. This may be done by a series of treatments that will vary somewhat from one installation to another. One such series is air from the outside first is passed through a prefilter, usually of glass wool, cloth or shredded plastic, to remove large particles. Then it is treated by passage through an electrostatic precipitator (Suppliers: *Am Air, Electro-Air, Sturtevant*). Such a unit induces an electrical charge on particles in the air and removes them by attraction to oppositely charged plates. The air then passes through the most efficient cleaning device, a HEPA (high efficiency particulate air) filter having an efficiency of at least 99.97% in removing particles of 0.3 μm and larger, based on the DOP (Diocetyl phthalate) test (Suppliers: *Am Air, Cambridge, Flanders*).

For personnel comfort, air conditioning and humidity control should be incorporated into the system. Another system, the Kathabar system (*Surface Combustion*), cleans the air of dirt and microorganisms by washing it in an antiseptic solution and, at the same time, controls the humidity. The clean, aseptic air is introduced into the aseptic area and maintained under positive pressure, which prevents outside air from rushing into the aseptic area through cracks, temporarily open doors or other openings.

Laminar-Flow Environments—The required environmental control of aseptic areas has been made possible by the use of laminar airflow enclosures. Laminar airflow provides a total sweep of a confined space because the entire body of air moves with a uniform velocity, usually 90 ft/min, \pm 20% along parallel lines, originating through a HEPA filter occupying one entire side of the confined space. Therefore, it bathes the total space with very clean air, sweeping away contaminants.

The arrangement for the direction of airflow can be horizontal (see Fig 9) or vertical (see Fig 10), and may involve a limited area such as a workbench or an entire room.

Figure 10 shows a vial-filling line protected with vertical laminar airflow from ceiling-hung HEPA filters. Plastic curtains are installed to maintain the laminarity of airflow to below the filling line and to circumscribe the critical filling portion of the line. The area outside the curtains can be

Fig 1
Sanit

main
insu
L
stan
Star
tain
size
Clas
10,
acc
uct
mer
clea
It
ups
filt
flow
ope
exa
mic
Fur
con
hor
con
the
thro
for
for

Fig
enc

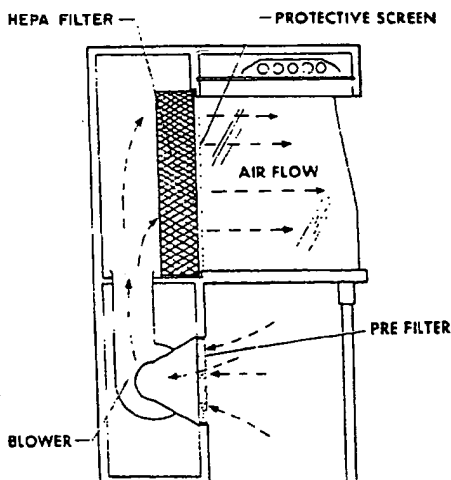


Fig 9. Horizontal laminar-flow workbench (courtesy, adaptation, Sandia).

maintained at a slightly lower level of cleanliness than that inside.

Laminar flow of HEPA-filtered air is capable of meeting the standard for a Class 100 clean room as defined by Federal Standard 209E,¹⁶ which states that such an environment contains no more than 100 particles/ft³ of 0.5 μ m and larger in size. Thus, in Fig 10, the area within the curtains should be a Class 100 environment but the area outside may be Class 10,000 or cleaner, defined on the same basis. Today, it is accepted that critical areas of processing, wherein the product or product contact surfaces may be exposed to the environment, even for a brief period of time, should meet Class 100 clean room standards.

It must be borne in mind that any contamination introduced upstream by equipment, arms of the operator or leaks in the filter will be blown downstream. In the instance of horizontal flow this may be to the critical working site, the face of the operator or across the room. Should the contaminant be, for example, penicillin powder, a biohazard material or viable microorganisms, the danger to the operator is apparent. Further, great care must be exercised to prevent cross-contamination from one operation to another, especially with horizontal laminar air flow. For operations involving such contaminants a vertical system is much more desirable, with the air flowing through perforations in the countertop or through return louvers at floor level, where it can be directed for decontamination. Vertical flow has been recommended for sterility-testing procedures.

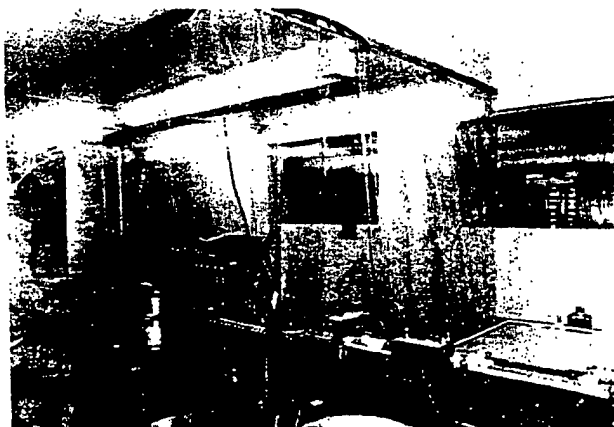


Fig 10. Vial filling line under vertical laminar airflow with critical area enclosed within plastic curtains (courtesy, Merck).

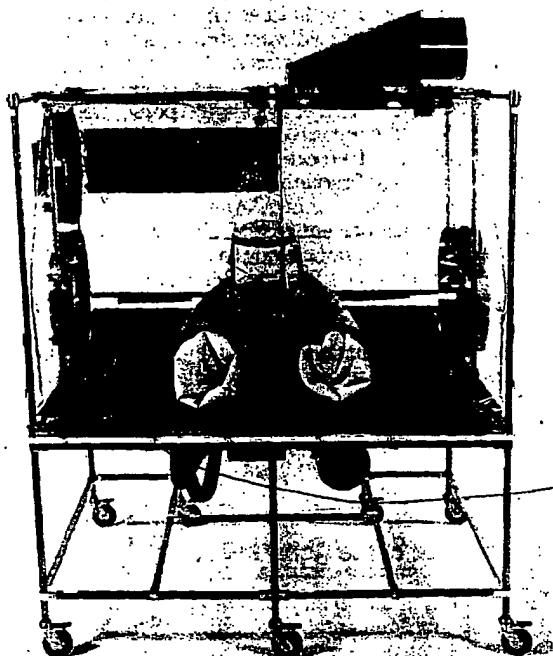


Fig 11. One configuration of an isolator (courtesy, Amsco).

Laminar-flow environments provide well-controlled work areas only if proper precautions are observed. Any reverse air currents or movements exceeding the velocity of the HEPA-filtered airflow may introduce contamination, as may coughing, reaching or other manipulations of operators.

Therefore, laminar-flow work areas should be protected by being located within controlled environments. Personnel should be attired for aseptic processing as described below. All movements and processes should be planned carefully to avoid the introduction of contamination upstream of the critical work area. Checks of the airstream should be performed initially and at regular intervals to be sure no leaks have developed through or around the HEPA filters.

Workbenches and other types of laminar-flow enclosures are available from several commercial sources (Suppliers: Air Control, Atmos-Tech, Baker, Clean Air, Clestru, EACI/Enviroco, Flanders, Laminair, Liberty, Veco).

Isolation (barrier) technology is a relatively new approach designed to isolate aseptic operations from personnel. Figure 11 shows the structure of one configuration of an isolator. As can be seen, the operations are performed within transparent, plastic, sealed walls with the operator, in this instance, working through gloves and a half-suit. These isolation work stations are presterilized, usually with peracetic acid or hydrogen peroxide vapor, and sterile supplies are introduced from sterilizing modules through uniquely engineered transfer ports. Results from the use of these units in controlling contamination during sterility testing have reduced false positives to essentially zero. Studies are being undertaken to evaluate these units for aseptic production operations.¹⁷

Ultraviolet Radiation

Ultraviolet (UV) light rays have an antibacterial action, thereby producing a disinfectant action on directly irradiated surfaces. Since these rays cannot penetrate most materials,

only a surface effect is produced, with the principal exception being limited penetration through air and pure water. UV light rays travel in straight lines only; therefore, objects in the path of the light beam will cast shadows with a resultant lack of irradiation in the shadow area.

UV rays are irritating to the skin and, particularly, the eyes of human beings. Therefore, should personnel be in the area of irradiation, they must be protected from direct exposure. Direct irradiation of a room when personnel are not present is a valuable means of reducing the bacterial count on working surfaces and floors.

The best practical source of UV light rays is the cold-cathode mercury vapor lamp. This lamp emits a high proportion of radiation at the 253.7 nm wavelength. A special glass is used for the tube so that the rays will pass to the outside. This glass gradually will change in crystal structure with use so that passage of the rays is gradually reduced. Such lamps, therefore, rarely burn out as do visible-light lamps but gradually reach an emission level which is ineffective. These lamps also must be kept clean, for dust and grease will lower the effective emission drastically. It generally is stated that an irradiation intensity of $20 \mu\text{w}/\text{cm}^2$ is required for effective antibacterial activity.

Maintenance of the Aseptic Area

Housekeeping and maintenance are important aspects in controlling environmental contamination in the aseptic area. These should be done by crews given special instruction and under the supervision of personnel trained in the care of such areas. In general, cleaning and maintenance should be done after the completion of the day's work with an interval of quietude before the beginning of another aseptic operation. With the advent of laminar flow of HEPA-filtered air the rigors of cleaning have been reduced since the clean airflow continuously "sweeps" the area clean. All maintenance equipment should be selected for its effectiveness and freedom from lint-producing tendencies and should be reserved for use in aseptic areas only.

Personnel

Personnel selected to work on the preparation of a parenteral product must be neat, orderly and reliable. They should be in good health and free from dermatological conditions that might increase the microbial load. If they show symptoms of a head cold, allergies or similar illness, they should not be permitted in the aseptic area until their recovery is complete. However, a healthy person with the best personal hygiene still will shed large numbers of viable and nonviable particles from body surfaces. This natural phenomenon creates continuing problems when personnel are present in clean rooms, but effective training and proper gowning can reduce, but not eliminate, the problem of particle shedding from personnel.

Aseptic-area operators should be given thorough, formal training in the principles of aseptic processing and the techniques to be employed. Subsequently, the acquired knowledge and skills should be evaluated, to be sure training has been effective, before they are allowed to participate in the preparation of sterile products. Retraining should be performed on a regular schedule to enhance the maintenance of the required level of expertise. An effort should be made to imbue operators with an awareness of the vital role they play in determining the reliability and safety of the final product. This is especially true of supervisors since they should be individuals who not only understand the unique requirements of aseptic procedures but who are able to obtain the full participation of other employees in fulfilling these exacting requirements.

The uniform worn is designed to confine the contaminants discharged from the body of the operator, thereby preventing

their entry into the production environment. For use in the aseptic area, uniforms should be sterile. Fresh, sterile uniforms should be used after every break period, or whenever the individual returns to the aseptic area. In some plants this is not required if the product is to be sterilized in its final container. The uniform usually consists of coveralls for both men and women, hoods to completely cover the hair, face masks and Dacron or plastic boots (Fig 12). Sterile rubber gloves also are required for aseptic operations, preceded by thorough scrubbing of the hands with a disinfectant soap. In addition, goggles may be required to complete the coverage of all skin areas.

Dacron or Tyvek uniforms are used usually, are effective barriers to discharged body particles (viable and nonviable), are essentially lint-free and are reasonably comfortable. Air showers are sometimes directed on personnel entering the processing area to blow loose lint from the uniforms.

Environmental Control Evaluation

As evidenced by the above discussion, manufacturers of sterile products use extensive means to control the environment so that these critical products can be prepared free from contamination. Nevertheless, tests should be performed to determine the level of control actually achieved. Normally, the tests consist of counting viable and nonviable particles suspended in the air or settled on surfaces in the workspace. A baseline count, determined by averaging multiple counts when the facility is operating under controlled conditions, is used to establish the optimal test results expected. During the subsequent monitoring program, the test results are followed carefully for high individual counts, a rising trend or other abnormalities. If they exceed selected alert or action levels, a plan of action must be put into operation to determine if or what corrective measures are required.

The tests used generally measure either the particles in a volume of sampled air or the particles that are settling or are present on surfaces. A volume of air measured by an elec-



Fig 12. Appropriate uniform for operators entering an aseptic filling room (courtesy, Abbott).

tronic particle counter will detect all particles instantly, but not differentiate between viable and nonviable ones. However, because of the need to control the level of microorganisms in the environment in which sterile products are processed, it also is necessary to detect viable particles. These usually are less in number than nonviable ones and are only detectable as colony forming units (CFUs) after a suitable incubation period at, for example, 30 to 35°C for up to 48 hours.

Locations for sampling should be planned to reveal potential contamination levels which may be critical in the control of the environment. For example, the most critical process step is usually the filling of dispensing containers, a site obviously requiring monitoring. Other examples include the gowning room, high-traffic sites in and out of the filling area, the penetration of conveyor lines through walls and sites near the inlet and exit of the air system.

The size of the sample should be large enough to obtain a meaningful particle count. At sites where the count is expected to be low the size of the sample may need to be increased; for example, in Class 100 areas, Whyte and Niven,¹⁸ suggest that the sample should be at least 30 ft³ and, probably, much more. They also suggest that settling plates should be exposed in Class 100 areas for an entire fill (up to 7 to 8 hours) rather than the more common 1 hour. However, excessive dehydration of the medium must be avoided, particularly in the path of laminar-flow air.

To measure the total particle content in an air sample, electronic particle counters are available, operating on the principle of the measurement of light-scattered from particles as they pass through the cell of the optical system (Suppliers: *Climet, Met One, Particle Measuring, Royco*). These instruments not only count particles but also provide a size distribution based on the magnitude of the light scattered from the particle.

Several air-sampling devices are used to obtain a count of microorganisms in a measured volume of air. A slit-to-agar (STA) sampler (Suppliers: *Mattson-Garvin, New Brunswick*) draws by vacuum a measured volume of air through a narrow opening causing the air to impact on the surface of a slowly rotating nutrient agar plate. Microorganisms adhere to the surface of the agar and grow into visible colonies which are counted as CFUs, since it is not known whether the colonies arise from a single microorganism or a cluster. A centrifugal sampler (Supplier: *Biotest*) pulls air into the sampler by means of a rotating propeller and slings the air by centrifugal action against a peripheral nutrient agar strip. The advantages of this unit are that it can be disinfected easily and is portable so that it can be hand-carried wherever needed. These two methods are used quite widely.

A widely used method for microbiological sampling consists of the exposure of nutrient agar culture plates to the settling of microorganisms from the air. This method is very simple and inexpensive to perform but will detect only those organisms which have settled on the plate; therefore, it does not measure the number of microorganisms in a measured volume of air. Nevertheless, if the conditions of exposure are repeated consistently, a comparison of CFUs at one sampling site from one time to another can be meaningful.

The number of microorganisms on surfaces can be determined with nutrient agar plates having a convex surface (*Rodac Plates*). With these it is possible to roll the raised agar surface over flat or irregular surfaces to be tested. Organisms will be picked up on the agar and will grow during subsequent incubation. This method also can be used to assess the number of microorganisms present on the surface of the uniforms of operators, either as an evaluation of gowning technique immediately after gowning or as a measure of the accumulation of microorganisms during processing. Whenever used, care must be taken to remove any agar residue left on the surface tested.

Further discussion of proposed viable particle test methods and the counts to be accepted will be found in Section (1116) "Microbial Evaluation and Classification of Clean Rooms and Clean Zones" in *Pharm Forum* 18:4048, 1992.

Results from the above tests are very valuable to keep cleaning, production and quality-control personnel apprised of the level of contamination in a given area and, by comparison with baseline counts, will indicate when more extensive cleaning and sanitizing is needed. The results also may serve to detect environmental control defects such as failure in air-cleaning equipment or the presence of personnel who may be disseminating large numbers of bacteria without apparent physical ill effects.

Media Fill—An evaluation which is not strictly an environmental test, but which includes an evaluation of the environment along with the process, the operators and the equipment, is the "media fill." Sterile trypticase soy broth is filled into sterile containers under conditions simulating as closely as possible those characteristics of a filling process for a product. The entire lot, normally at least 3000 units, is then incubated at a suitable temperature, usually 20 to 25°, for at least 14 days and examined for the appearance of growth of microorganisms. If growth occurs, contamination has entered the container(s) during the processing. To pass the test not more than 0.1% of the units may show growth. This evaluation also has been used as a measure of the proficiency of an individual operator. This test is a very stringent evaluation of the efficiency of an aseptic filling process and, by many, is considered to be the most evaluative test available.

Production Procedures

The processes required for preparing sterile products constitute a series of events initiated with the securing of approved raw materials (drugs, excipients, vehicles, etc) and primary packaging components (containers, closures, etc) and ends with the sterile product sealed in its dispensing package. Each step in the process must be controlled very carefully in order that the product will have its required quality. To assure the latter, each process should be validated to be sure that it is accomplishing what it is intended to do. For example, an autoclave sterilization process must be validated by providing data showing that it effectively kills resistant forms of microorganisms; or, a cleaning process for rubber closures should provide evidence that it is cleaning closures to the required level of cleanliness. The validation of processes requires an extensive and intensive effort to be successful, and is an integral part of CGMP requirements.

In the following sections the production procedures used in preparing sterile drug products will be discussed.

Cleaning Containers and Equipment

Containers and equipment coming in contact with parenteral preparations must be cleaned meticulously. It is obvious that if this were not so, all other precautions to prevent contamination of the product would be useless. It also should be obvious that even new, unused containers and equipment will be contaminated with such debris as dust, fibers, chemical films and other materials arising from such sources as the atmosphere, cartons, the manufacturing process and human hands. Much greater contamination must be removed from previously used equipment before it will be suitable for reuse. Equipment should be reserved exclusively for use only with

parenteral preparations and, where conditions dictate, only for one product in order to reduce the risk of contamination.

A variety of machines are available for cleaning containers for parenteral products. These vary in complexity from a small rotary rinser (Fig 13) to large automatic washers capable of processing several thousand containers an hour (Fig 14). The selection of the particular type will be determined largely by the physical type of containers, their condition with respect to contamination and the number to be processed in a given period of time.

Characteristics of Machinery—Regardless of the type of cleaning machine selected, certain fundamental characteristics usually are required.

1. The liquid or air treatment must be introduced in such a manner that it will strike the bottom of the inside of the inverted container, spread in all directions and smoothly flow down the walls and out the opening with a sweeping action. The pressure of the jet stream should be such that there is minimal splashing, and the flow should be such that it can leave the container opening without accumulating and producing turbulence inside. Splashing may prevent cleaning all areas, and turbulence may redeposit loosened debris. Therefore, direct introduction of the jet stream within the container with control of its flow is required.

2. The container must receive a concurrent outside rinse.

3. The cycle of treatment should provide for a planned sequence alternating very hot and cool treatments. The final treatment should be an effective rinse with water of a quality equivalent to WFI.

4. All metal parts coming in contact with the containers and with the treatments should be constructed of stainless steel or some other noncorroding and noncontaminating material.

Treatment Cycle—The cycle of treatments to be employed will vary with the condition of the containers to be cleaned. In general, loose dirt can be removed by vigorous rinsing with water. Detergents rarely are used for new containers because of the risk of leaving detergent residues. However, a thermal-shock sequence in the cycle usually is employed to aid, by expansion and contraction, loosening of debris that may be adhering to the container wall. Sometimes only an air rinse is used for new containers, particularly if used for a dry powder. In all instances the final rinse, whether air or WFI, must be ultraclean so that no particulate residues are left by the rinsing agent.

Only new containers are used for parenterals. Improvements have been made in maintaining their cleanliness during shipment from the manufacturer through tight, low-shedding packaging, including plastic blister packs, as can be seen stacked on the right of Fig 14.

Machinery for Containers—The machinery available for cleaning containers embodies the above principles but varies in the mechanics by which it is accomplished. In one approach, the jet tubes are arranged on arms like the spokes of a wheel, which rotate around a center post through which the



Fig 13. Rotary rinser (Cozzoli) in a clean environment provided by vertical laminar airflow within a curtained enclosure (courtesy, Ciba-Geigy).

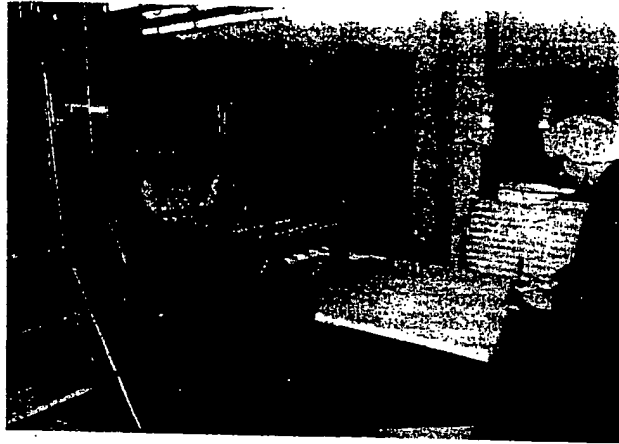


Fig 14. Loading end of large conveyor vial washer that subjects inverted vials to a series of cleaning steps before delivery from the far end of the washer. Note the vials in plastic blister packs at right of operator (courtesy, Merck).

treatments are introduced. An operator places the unclean containers on the jet tubes as they pass the loading point and removes the clean containers as they complete one rotation. Such a small-scale machine is pictured in Fig 14. A washer capable of cleaning hundreds of containers an hour, shown in Figure 14, uses a row of jet tubes across a conveyor belt. The belt moves the inverted containers past the programmed series of treatments and discharges the clean containers into a sterilizing oven (not shown), which ultimately discharges them through a wall into a clean room for filling. Another type of machine is the rack-loading washer. Stainless-steel racks are designed to fit over the open ends of ampuls or vials as configured in trays of shipping cartons or blister packs. Inverting the trays permits the containers to slide into the racks so that they can be handled by the quantity in the tray, as shown in Fig 15. The clean containers may be transferred directly to the conveyor of a sterilizing tunnel (as shown), or they may be placed in stainless-steel boxes for subsequent dry-heat sterilization and storage. A continuous automated line operation is shown in Figure 16. The vials are fed into the rotary rinser in the foreground, transferred automatically to the covered sterilizing tunnel in the center, conveyed through the wall in the background and discharged through the wall into the filling clean room.

Handling after Cleaning—The wet, clean containers must



Fig 15. Cleaning vials with rack-loading washer, permitting handling vials by a full rack. After multiple-washing treatments, the racks are placed directly on the conveyor belt of the hot-air sterilizing tunnel (courtesy, Merck).

Fig 1
rinse
tion

be l
rein
mor
rins
of c
with
each
prot
gro
con
afte
bee
the
dat

Ir
mer
mu
trol
the
flov
deli
C
the
ele
"bl
pot
for
The
tim
WF
aw
tec
iliz
unt
mu
fro
ou
aut
im
for
the
va

is
wa
the
in
flu
tic

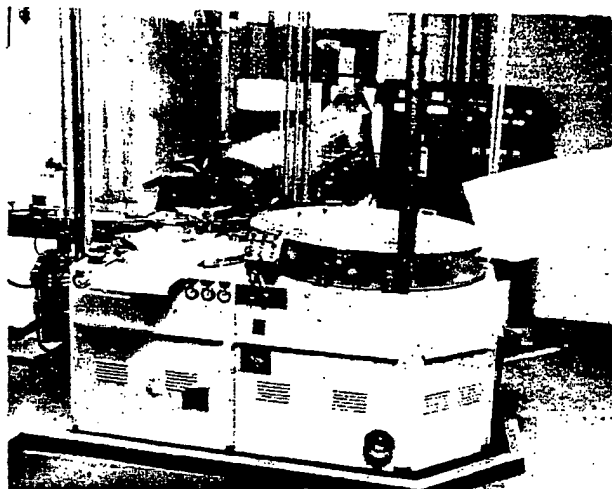


Fig 16. Continuous automatic line operation for vials from a rotary rinser through a sterilizing tunnel with vertical laminar-airflow protection of clean vials (courtesy, Abbott).

be handled in such a way that contamination will not be reintroduced. A wet surface will collect contaminants much more readily than will a dry surface. For this reason wet, rinsed containers must be protected, such as by a laminar flow of clean air until covered, as within a stainless-steel box or within a sterilizing tunnel. Although not clearly visible in each instance, the wet, clean containers in Figs 13–16 were so protected. In addition, microorganisms are more likely to grow in the presence of moisture. Therefore, wet, clean containers should be dry-heat sterilized as soon as possible after washing. Doubling the heating period generally has been considered to be adequate also to destroy pyrogens, but the actual time-temperature conditions required must be validated.

Increases in process rates have necessitated the development of continuous, automated line processing with a minimum of individual handling, still maintaining adequate control of the cleaning and handling of the containers. In Fig 16, the clean, wet containers are protected by filtered laminar-flow air from the rinser through the tunnel and until they are delivered to the filling line.

Closures—Rubber closures are coated with lubricant from the molding operation. In addition, the rough surface and electrostatic attraction tend to hold debris. Also, the surface "bloom" from migrated inorganic constituents of the compound must be removed. The recommended procedure calls for gentle agitation in a hot solution of a mild water softener. The closures are removed from the solution and rinsed several times, or continuously for a prolonged period, with filtered WFI. The rinsing is to be done in a manner which will flush away loosened debris. The wet closures are carefully protected from pick up of environmental contamination, are sterilized, usually by autoclaving, and stored in closed containers until ready for use. This cleaning and sterilizing process must be validated with respect to rendering the closures free from pyrogens. Actually, it is the cleaning and final, thorough rinsing with WFI that must remove pyrogens, since autoclaving does not destroy them. If the closures were immersed during autoclaving, the solution is drained off before storage to reduce hydration of the rubber compound. If the closures must be dry for use, they may be subjected to vacuum drying at a temperature in the vicinity of 100°.

The equipment used for washing large numbers of closures is usually an agitator or horizontal basket-type automatic washing machine. Because of particulate generation from the abrading action of these machines, some heat the closures in kettles in detergent solution and follow with prolonged flush rinsing. The final rinse always should be with low particulate WFI.

Equipment—The details of certain prescribed techniques for cleaning and preparing equipment, as well as of containers and closures, have been presented elsewhere.¹⁹ Here, a few points will be emphasized.

All equipment should be disassembled as much as possible to provide access to internal structures. For thorough cleaning, surfaces should be scrubbed thoroughly with a stiff brush using an effective detergent, paying particular attention to joints, crevices, screw threads and other structures where debris is apt to collect. Exposure to a stream of clean steam will aid in dislodging residues from the walls of stationary tanks, spigots, pipes and similar structures. Thorough rinsing with distilled water should follow the cleaning steps. Large stationary tanks, such as those shown in Fig 17, should be protected as much as possible from contamination after cleaning but should be rinsed thoroughly again with distilled water or WFI prior to reuse.

Because of the inherent variation in the manual cleaning of tanks and other large equipment items, together with the need to validate the process, an automated, usually computer-controlled, system has been developed called "cleaning in place" (CIP).²⁰ Such an approach involves designing the system, normally of stainless steel, with smooth, rounded internal surfaces and without crevices. That is, for example, with welded rather than threaded connections. The cleaning is accomplished with the scrubbing action of high-pressure spray balls or nozzles delivering hot detergent solution from tanks captive to the system. Thorough rinsing with WFI follows and is accomplished within the same system. The system often is extended to permit sterilizing in place (SIP) as well.²¹

Rubber tubing, rubber gaskets and other rubber parts may be washed in a manner such as described for rubber closures. Thorough rinsing of tubing must be done by passing WFI through it. However, due to the relatively porous nature of rubber compounds and the difficulty in removing all traces of chemicals from previous use, it is considered by some inadvisable to reuse rubber tubing. Rubber tubing must be left wet when preparing for sterilization by autoclaving.

Product Preparation

The basic principles employed in the compounding of the product do not vary from those used routinely by qualified pharmacists. However, selected aspects will be mentioned for emphasis.

A master formula would have been developed and on file. Each batch formula sheet should be prepared from the master and confirmed for accuracy. All measurements of quantities

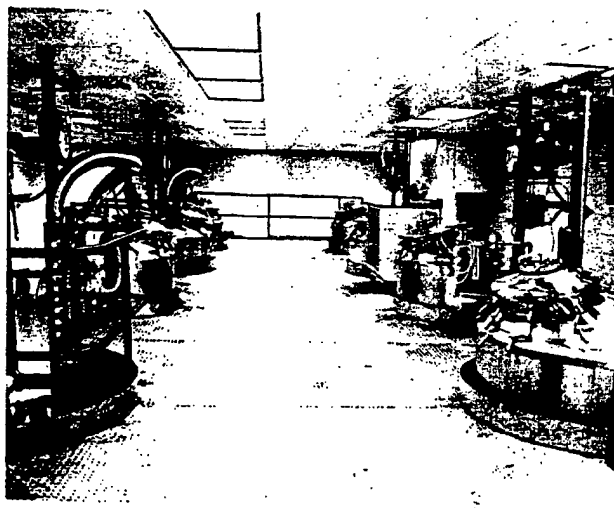


Fig 17. Large stainless-steel tanks for product preparation showing mezzanine access level (courtesy, Abbott).

should be made as accurately as possible and checked by a second qualified person. Frequently, today, the formula documents are generated by a computer and the measurements of quantities of ingredients computer controlled. Although most liquid preparations are dispensed by volume, they are prepared by weight, since weighings can be performed more accurately than volume measurements and no consideration needs to be given to the temperature.

Care must be taken that equipment is not wet enough to significantly dilute the product or, in the case of anhydrous products, to cause a physical incompatibility. The order of mixing of ingredients may affect the product significantly, particularly those of large volume where attaining homogeneity requires considerable mixing time. For example, the adjustment of pH by the addition of an acid, even though diluted, may cause excessive local reduction in the pH of the product so that adverse effects are produced before the acid can be dispersed throughout the entire volume of product.

Parenteral dispersions, including colloids, emulsions and suspensions, provide particular problems. Parenteral emulsions have been reviewed by Singh and Ravin.²² In addition to the problems of achieving and maintaining proper reduction in particle size under aseptic conditions, the dispersion must be kept in a uniform state of suspension throughout the preparative, transfer and subdividing operations.

The formulation of a stable product is of paramount importance. Certain aspects of this have been mentioned in the discussion of components of the product. Exhaustive coverage of the topic is not possible within the limits of this text, but further coverage is provided in Chapter 83. It should be mentioned here, however, the thermal sterilization of parenteral products increases the possibility of chemical reactions. Such reactions may progress to completion during the period of elevated temperature in the autoclave, or be initiated at this time but continue during subsequent storage. The assurance of attaining product stability requires a high order of pharmaceutical knowledge and responsibility.

Filtration

After a product has been compounded, it must be filtered if it is a solution. The primary objective of filtration is to clarify a solution. A high degree of clarification is termed "polishing" a solution. This term is used when particulate matter down to approximately 2 μm in size is removed. A further step, removing particulate matter down to 0.2 μm in size, would eliminate microorganisms and would accomplish "cold" sterilization. A solution having a high degree of clarity conveys the impression of high quality and purity, desirable characteristics for a parenteral solution.

Filters are thought to function by one or, usually, a combination of the following: (1) sieving or screening, (2) entrapment or impaction and (3) electrostatic attraction. When a filter retains particles by sieving, they are retained on the surface of the filter. Entrapment occurs when a particle, smaller than the dimensions of the passageway (pore), becomes lodged in a turn or impacted on the surface of the passageway. Electrostatic attraction causes particles opposite in charge to that of the surface of the filter pore to be held or adsorbed to the surface. It should be noted that increasing, prolonging or varying the force behind the solution may tend to sweep particles initially held by entrapment or electrostatic charge through the pores and into the filtrate.

Membrane filters are used for parenteral solutions because of their particle-retention effectiveness, nonshedding property, nonreactivity and disposable characteristics. However, it should be noted that nonreactivity does not apply in all cases. For example, polypeptide products may show considerable adsorption through some membrane filters, but those composed of polysulfone and polyvinylidene difluoride have been developed to be essentially nonadsorptive for these products. The most common membranes are composed of

Cellulose ester (Suppliers: *Gelman, Millipore, Sartorius, Schleicher-Seitz*).

Nylon (Supplier: *Pall*).

Polysulfone (Supplier: *Gelman*).

Polycarbonate (Supplier: *Nuclepore*).

Polyvinylidene difluoride (Supplier: *Millipore*).

Polytetrafluoroethylene (Teflon) (Supplier: *Millipore*).

They are available as flat membranes or pleated into cylinders to increase surface area and, thus, flow rate. Each filter in its holder should be tested for integrity before and after use, particularly if it is being used to eliminate microorganisms. This integrity test usually is performed as the *bubble-point test*, a test to detect the largest pore, or other opening, through the membrane. The basic test is performed by gradually raising air pressure on the upstream side of a water-wet filter. The pressure at which bubbles first appear downstream is the bubble point. This pressure is characteristic for each pore size of a filter and is provided by the filter manufacturer. For example, a 0.2 μm cellulose ester filter will bubble at about 50 psig. If the filter is wetted with other liquids, such as a product, the bubble point will differ and must be determined experimentally. If the bubble point is lower than the rated pressure, the filter is defective, probably due to a puncture or tear, and should not be used. As the surface area of filters becomes large, diffusion of air through the water-filled pores tends to obscure the bubble point. Therefore, a diffusion, or pressure hold, test has been developed as an integrity test for filters with large surface areas. Particulars are obtainable from the filter manufacturer.

While membrane filters are disposable, and thus discarded after use, the holders must be cleaned thoroughly between uses. Today, clean, sterile, pretested, disposable assemblies for small as well as large volumes of solutions are available commercially. Other characteristics of these filters, important for a full understanding of their use, are given in Chapter 84 and in a review article.²³

Filling

During the filling of containers with a product, the most stringent requirements must be exercised to prevent contamination, particularly if the product has been sterilized by filtration and will not be sterilized in the final container. Under the latter conditions the process usually is called an "aseptic fill" and is validated with media fills (see page 1537). During the filling operation, the product must be transferred from a bulk container and subdivided into dose containers. This operation exposes the sterile product to the environment, equipment and manipulative technique of the operator until it can be sealed in the dose container. Therefore, this operation is carried out with a minimum exposure time in the aseptic filling area where maximum protection is provided. Additional protection may be provided by filling under a blanket of HEPA-filtered laminar-flow air within the aseptic area.

Normally, the compounded product is in the form of either a liquid or a solid. A liquid is more readily subdivided uniformly and introduced into a container having a narrow mouth than is a solid. Mobile, nonsticking liquids are considerably easier to transfer and subdivide than viscous, sticky liquids, which require heavy-duty machinery for rapid production filling.

Although many devices are available for filling containers with liquids, certain characteristics are fundamental to them all. A means is provided for repetitively forcing a measured volume of the liquid through the orifice of a delivery tube which is introduced into the container. The size of the delivery tube will vary from that of about a 20-gauge hypodermic needle to a tube $\frac{1}{2}$ in. or more in diameter. The size required is determined by the physical characteristics of the liquid, the desired delivery speed and the inside diameter of the neck of the container. The tube must enter the neck and deliver the liquid well into the neck to eliminate spillage, allowing sufficient clearance to permit air to leave the container as the liquid enters. The delivery tube should be as large in diameter as possible in order to reduce the resistance to the flow of the liquid. For smaller volumes of liquids, the delivery usu-

ally is
forci
nate
slidin
Other
funne
volur
cont
to tri
pump
Th
betw
drop
after
tube
fillin
can t
Sh
the n
of ne
steel
clea
Be
prep
twee
Most
appr
This
bran
inse
mati
be f
from
final
fite
drop
mak
wou
Li
be a
liqu
nee
spe
This
and
san
disj

ally is obtained from the stroke of the plunger of a syringe, forcing the liquid through a two-way valve providing for alternate filling of the syringe and delivery of mobile liquids. A sliding piston valve would be used for heavy, viscous liquids. Other mechanisms include the turn of an auger in the neck of a funnel or the oscillation of a rubber diaphragm. For large volumes the quantity delivered usually is measured in the container by the level of fill in the container, the force required to transfer the liquid being provided by gravity, a pressure pump or a vacuum pump.

The narrow neck of an ampul limits the clearance possible between the delivery tube and the inside of the neck. Since a drop of liquid normally hangs at the tip of the delivery tube after a delivery, the neck of an ampul will be wet as the delivery tube is withdrawn, unless the drop is retracted. Therefore, filling machines should have a mechanism by which this drop can be drawn back into the lumen of the tube.

Since the liquid will be in intimate contact with the parts of the machine through which it flows, these must be constructed of nonreactive materials such as borosilicate glass or stainless steel. In addition, they should easily be demountable for cleaning and sterilization.

Because of the concern for particulate matter in injectable preparations, a final filter often is inserted in the system between the filler and the delivery tube, as shown in Fig 18. Most frequently this is a membrane filter, having a porosity of approximately 1 μm and treated to have a hydrophobic edge. This is necessary to reduce the risk of rupture of the membrane due to filling pulsations. It should be noted that the insertion of the filter at this point should collect all particulate matter generated during the process. Only that which may be found in inadequately cleaned containers or picked up from exposure to the environment after passage through the final filter potentially remain as contaminants. However, the filter does cushion liquid flow and reduces the efficiency of drop retraction from the end of the delivery tube, sometimes making it difficult to control delivery volume as precisely as would be possible without the filter.

Liquids—The filling of a small number of containers may be accomplished with a hypodermic syringe and needle, the liquid being drawn into the syringe and forced through the needle into the container. A device for providing greater speed of filling is the Cornwall Pipet (*Becton Dickinson*). This has a two-way valve between the syringe and the needle and a means for setting the stroke of the syringe so that the same volume will be delivered each time. Clean, sterile, disposable assemblies (Suppliers: *Burron, Pharmaseal*) op-

erating on the same principle have particular usefulness in hospital pharmacy operations.

Mechanically operated instruments substitute a motor for the operator's hand in the previous devices described. Thereby, a much faster filling rate can be achieved. By careful engineering, the stroke of the syringe can be repeated precisely, and so, once a particular setting has been calibrated to the delivery, high delivery precision is possible. However, the speed of delivery, the expansion of the rubber tubing connecting the valve with the delivery tube and the rapidity of action of the valves can affect the precision of delivery. A filling machine employing a piston valve is shown in Fig 18. Stainless-steel syringes are required with viscous liquids because glass syringes are not strong enough to withstand the high pressures developed during delivery.

When high-speed filling rates are desired but accuracy and precision must be maintained, multiple filling units often are joined together in an electronically coordinated machine, such as shown in Fig 19. When the product is sensitive to metals, a peristaltic-pump filler may be used because the product comes in contact only with silicone rubber tubing. However, there is some sacrifice of filling accuracy.

Most high-speed fillers for large-volume solutions use the bottle as the measuring device, transferring the liquid either by vacuum or positive pressure from the bulk reservoir to the individual unit containers. Therefore, a high accuracy of fill is not achievable.

To insure delivery of the labeled volume to the patient, the USP provides a table of suggested fill volumes to include a slight excess over labeled volume.

Solids—Sterile solids, such as antibiotics, are more difficult to subdivide evenly into containers than are liquids. The rate of flow of solid material is slow and irregular. Even though a container with a larger diameter opening is used to facilitate filling, it is difficult to introduce the solid particles, and the risk of spillage is ever-present. The accuracy of the quantity delivered cannot be controlled as well as with liquids. Because of these factors, the tolerances permitted for the content of such containers must be relatively large. Suggested tolerances can be found in the USP.

Some sterile solids are subdivided into containers by individual weighing. A scoop usually is provided to aid in approximating the quantity required, but the quantity filled into the container finally is weighed on a balance. This is a slow process. When the solid is obtainable in a granular form so that it will flow more freely, other methods of filling may be employed. In general, these involve the measurement and delivery of a volume of the granular material which has been calibrated in terms of the weight desired. In the machine shown in Fig 20 an adjustable cavity in the rim of a wheel is

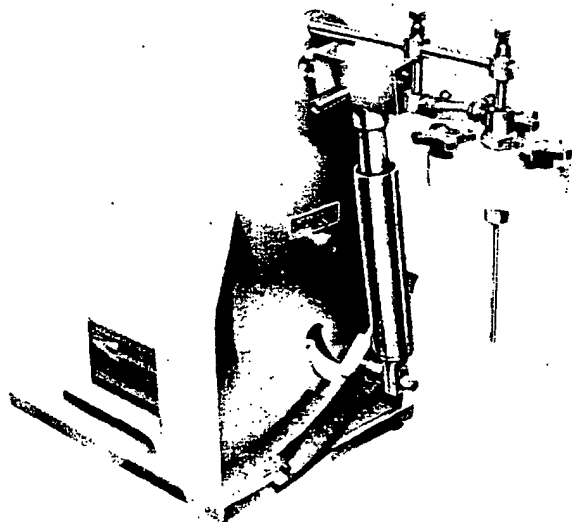


Fig 18. Filling machine employing a piston valve and a stainless-steel syringe (courtesy, Cozzoli).

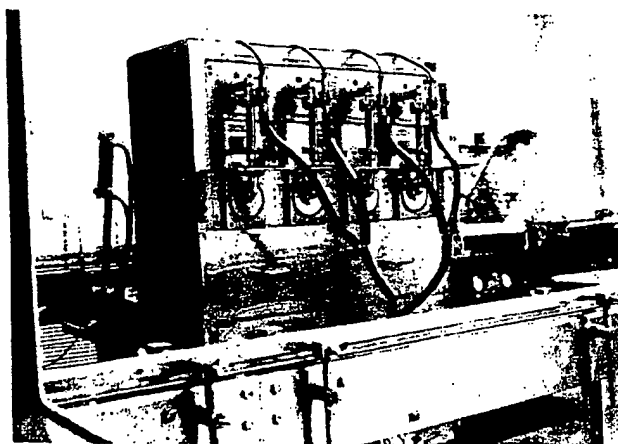


Fig 19. Four-pump liquid filler, with a conveyor line for vials protected by a vertical laminar airflow and plastic curtain; note the automatic stopping machine on the right within the curtain (courtesy, Abbott).

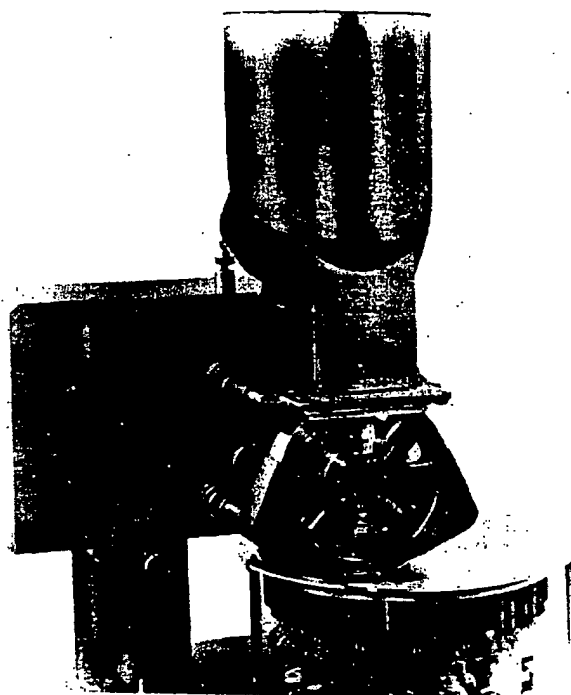


Fig 20. Accofil vacuum powder filler (courtesy, Perry).

filled by vacuum and the contents held by vacuum until the cavity is inverted over the container. The solid material then is discharged into the container by the use of sterile air. Another machine employs an auger in the stem of a funnel at the bottom of a hopper. The granular material is placed in the hopper. By controlling the size of the auger and its rotation, a regulated volume of granular material can be delivered from the funnel stem into the container. Such a machine is shown in Fig 21.

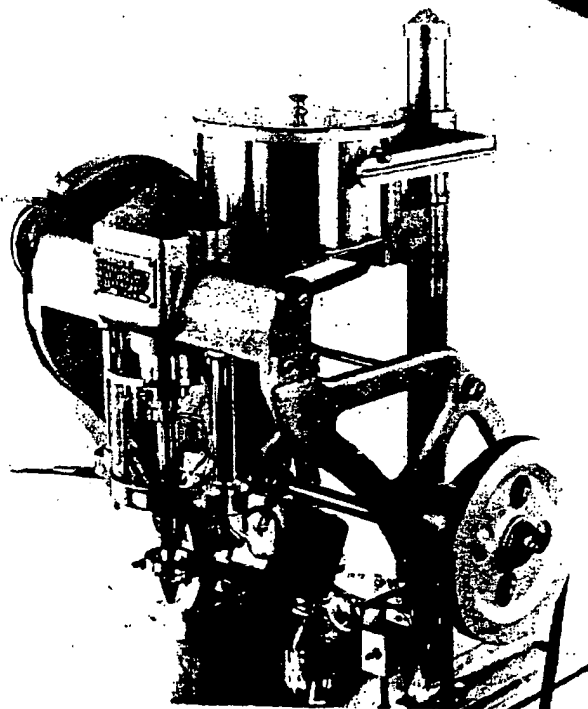


Fig 21. Auger-type powder filler (courtesy, Chase-Logeman).

Sealing

Ampuls—Filled containers should be sealed as soon as possible to prevent the contents from being contaminated by the environment. Ampuls are sealed by melting a portion of the glass neck. Two types of seals are employed normally: tip-seals (bead-seals) or pull-seals.

Tip-seals are made by melting enough glass at the tip of the neck of an ampul to form a bead and close the opening. These can be made rapidly in a high-temperature gas-oxygen flame. To produce a uniform bead, the ampul neck must be heated evenly on all sides. This may be accomplished by means of burners on opposite sides of stationary ampuls or by rotating the ampul in a single flame. Care must be taken to properly adjust the flame temperature and the interval of heating to obtain complete closing of the opening with a bead of glass. Excessive heating will result in the expansion of the gases within the ampul against the soft bead seal and cause a bubble to form. If it bursts, the ampul is no longer sealed; if it does not, the wall of the bubble will be thin and fragile. Insufficient heating will leave an open capillary through the center of the bead. An incompletely sealed ampul is called a "leaker."

Pull-seals are made by heating the neck of the ampul below the tip, leaving enough of the tip for grasping with forceps or other mechanical devices. The ampul is rotated in the flame from a single burner. When the glass has softened, the tip is grasped firmly and pulled quickly away from the body of the ampul, which continues to rotate. The small capillary tube thus formed is twisted closed. Pull-sealing is slower, but the seals are more sure than tip-sealing. Fig 22 shows a machine combining the steps of filling and pull-sealing ampuls.

Powder ampuls or other types having a wide opening must be sealed by pull-sealing. Were these sealed by tip-sealing, the very large bead produced would induce glass strain with subsequent fracture at the juncture of the bead and neck wall. Fracture of the neck of ampuls during sealing also may occur if wetting of the necks occurred at the time of filling. Also, wet necks increase the frequency of bubble formation. If the product in the ampul is organic in nature, wet necks also will result in unsightly carbon deposits from the heat of sealing.

In order to prevent decomposition of a product, it is sometimes necessary to displace the air in the space above the product in the ampul with an inert gas. This is done by introducing a stream of the gas, such as nitrogen or carbon dioxide, during or after filling with the product. Immediately thereafter the ampul is sealed before the gas can diffuse to the outside. This process should be validated to ensure adequate displacement of air by the gas in each container.

Vials and Bottles—These are sealed by closing the opening with a rubber closure (stopper). This must be accomplished as rapidly as possible after filling and with reasoned care to prevent contamination of the contents. The large opening makes the introduction of contamination much easier

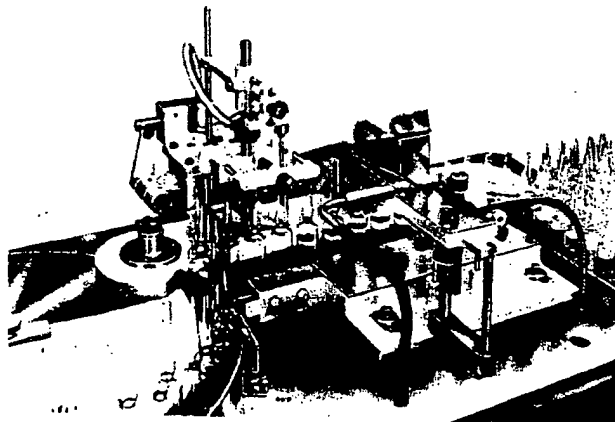


Fig 22. Automatic filling and pull-sealing of ampuls (courtesy, Cozzoli).

than
for s
fillin
Duri
prot
blan
and
TI
enoi
irreg
it m
necl
chai
spe
slidi
ing,
silic
it is
23).
with
risk
R
cap:
lip c
clos
cap
pro
unil
the
qua
T
dou
be r
dist
Rut
ofte
cas
is p

than with ampuls. Therefore, a covering should be provided for such containers except for the minimal time required for filling and for the actual introduction of the rubber closure. During the latter critical time the open containers should be protected from the ingress of contamination, preferably with a blanket of HEPA-filtered laminar airflow, as shown in Figs 10 and 19.

The closure must fit the mouth of the container snugly enough so that its elasticity will permit adjustment to slight irregularities in the lip and neck of the container. However, it must not fit so snugly that it is difficult to introduce into the neck of the container. Closures preferably are inserted mechanically using an automated process, especially with high-speed processing. To reduce friction so that the closure may slide more easily through a shute and into the container opening, the closure surfaces often are halogenated or treated with silicone. When the closure is positioned at the insertion site, it is mechanically pushed into the container opening (see Fig 23). When small lots are encountered, manual stoppering with forceps may be used, but such a process poses greater risk of introducing contamination than automated processes.

Rubber closures are held in place by means of aluminum caps. The caps cover the closure and are crimped under the lip of the vial or bottle to hold them in place (see Fig 5). The closure cannot be removed without destroying the aluminum cap; it is tamperproof. Therefore, an intact aluminum cap is proof that the closure has not been removed intentionally or unintentionally. Such confirmation is necessary to assure the integrity of the contents as to sterility and other aspects of quality.

The aluminum caps are so designed that the outer layer of double-layered caps, or the center of single-layered caps, can be removed to expose the center of the rubber closure without disturbing the band which holds the closure in the container. Rubber closures for use with intravenous administration sets often have a permanent hole through the closure. In such cases, a thin rubber disk overlaid with a solid aluminum disk is placed between an inner and outer aluminum cap, thereby

providing a seal of the hole through the closure. These are called triple-layered aluminum caps.

Single-layered aluminum caps may be applied by means of a hand crimper known as the Fermpress (Suppliers: *West, Wheaton*). Double- or triple-layered caps require greater force for crimping; therefore, heavy-duty mechanical crimpers (see Fig 24) are required (Suppliers: *Bosch, Cozzoli, Perry, West, Wheaton*).

Sterilization

Whenever possible, the parenteral product should be sterilized after being sealed in its final container (terminal sterilization) and within as short a time as possible after the filling and sealing have been completed. Since this usually involves a thermal process, due consideration must be given to the effect of the elevated temperature upon the stability of the product. Many products, both pharmaceutical and biological, will be affected adversely by the elevated temperatures required for thermal sterilization. Heat-labile products must, therefore, be sterilized by a nonthermal method, usually by filtration through bacteria-retaining filters. Subsequently, all operations must be carried out in an aseptic manner so that contamination will not be introduced into the filtrate. Colloids, oleaginous solutions, suspensions and emulsions that are thermolabile may require a process in which each component is sterilized separately and the product is formulated and processed under aseptic conditions.

The performance of an aseptic process is difficult and it has been accepted that currently, under the best controlled conditions, a sterility assurance level (SAL) of 10^{-3} is the maximum achievable. On the other hand, technical advances in aseptic processing, including improved automation, use of barrier systems, formulations to include antimicrobial effects and combinations of limited sterilization with aseptic processing, have improved the sterility-assurance levels achievable. Therefore, the successes realized should encourage continued efforts to improve the SAL achievable with aseptic processing. The importance of this is that for many drug solutions aseptic processing is the only method that can be considered for preparing a sterile product.

Nonthermal methods of sterilization, such as irradiation, have been proposed for consideration. However, since there is limited understanding of the molecular transformations that may occur in drug molecules and excipients under exposure to the high-energy levels of the process, extensive research will be required to develop the knowledge needed for an adequate evaluation.

Dry-heat sterilization may be employed for a few dry solids that are not affected adversely by the high temperatures and



Fig 23. Mechanical device for inserting rubber closures in vials (courtesy, Perry).

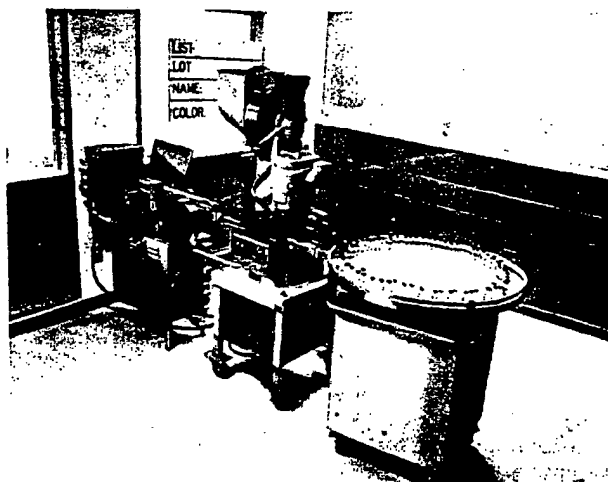


Fig 24. Applying aluminum caps to vials at the end of the process line (courtesy, Abbott).

for the relatively long heating period required. This method is applied most effectively to the sterilization of glassware and metalware. After sterilization, the equipment will be sterile, dry and, if the sterilization period is long enough, pyrogen-free.

Saturated steam under pressure (autoclaving) is the most commonly used and the most effective method for the sterilization of aqueous liquids or substances that can be reached or penetrated by steam. An SAL of 10^{-6} is readily achievable with terminal autoclaving of a thermally stable product. This is a 1000-fold improvement over the currently accepted level for aseptic processing. However, it needs to be noted that for terminal sterilization, as with autoclaving, the SAL is based upon an evaluation of the lethality of the process, i.e., of the probable number of viable microorganisms remaining in product units. However, for aseptic processing, the components used have been sterilized by a validated process and the SAL is based upon an evaluation of the probable number of product units that have been contaminated during the process. This difference does not alter the outcome but only the basis for evaluating the probable SAL.

Figure 25 shows liter containers of solution being loaded into an autoclave for sterilization. Since the temperature employed in an autoclave is lower than that for dry-heat sterilization, equipment made of materials such as rubber and polypropylene may be sterilized if the time and temperature are controlled carefully. As mentioned previously, some injections will be affected adversely by the elevated temperature required for autoclaving. For some products, such as Dextrose Injection, the use of an autoclave designed to permit a rapid rise to sterilizing temperature and rapid cooling with water spray after the sterilizing hold-period will make it possible to use this method. It is ineffective in anhydrous conditions, such as within a sealed ampul containing a dry solid or an anhydrous oil. Other products that will not withstand autoclaving temperatures may withstand marginal thermal methods such as tyndallization or inspissation. These methods may be rendered more effective for some injections by the inclusion of a bacteriostatic agent in the product.

It should be obvious that all materials subjected to sterilization must be protected from subsequent contamination to maintain their sterile state. Therefore, they must be wrapped or covered so that microorganisms may not gain access when removed from the autoclave. Equipment and supplies are wrapped most frequently with paper and tied or sealed with special autoclave tape. The wrapping must permit penetration of steam during autoclaving but screen out microorganisms when dry. A double wrapping with lint-free parchment paper designed for such use is probably best. Synthetic fiber

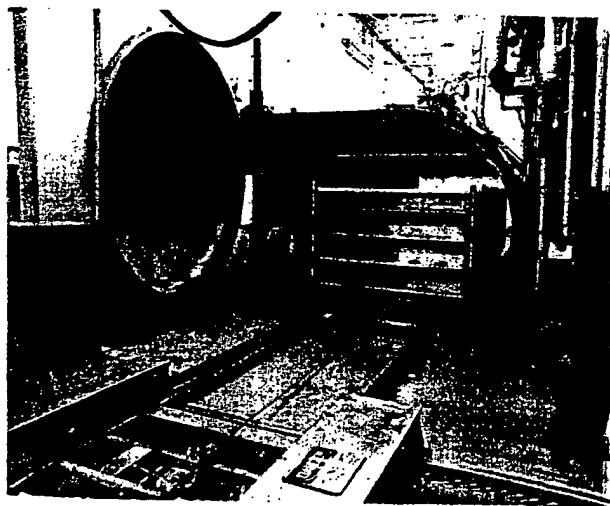


Fig 25. Large autoclave being loaded with liter bottles of parenteral solutions (courtesy, Abbott).

cloth such as nylon or Dacron also may be used for the inner wrapping. The openings of equipment subjected to dry-heat sterilization are often covered with silver-aluminum foil or with metal or glass covers. Cellulose wrapping materials are affected adversely by the high temperatures of dry-heat sterilization.

The effectiveness of any sterilization technique must be proved (validated) before it is employed. Since the goal of sterilization is to kill microorganisms, the ideal indicator to prove the effectiveness of the process is a resistant form of an appropriate microorganism, normally resistant spores (a biological indicator or BI). Therefore, during validation of a sterilization process, BIs of known resistance are used and numbers in association with physical-parameter indicators, such as recording thermocouples. Once the lethality of the process is established in association with the physical measurements, the physical measurements can be used for subsequent monitoring of in-use processes without the BIs. Eliminating the use of BIs in direct association with human-use products is appropriate because of the ever-present risk of an undetected, inadvertent contamination of a product or the environment.

The commercial suppliers of BIs have improved the resistance evaluation and lot-to-lot reliability of their products so that the information provided with each lot of BIs is generally reliable and useful in validation procedures.

In addition to the data printout from thermocouples, sometimes other physical indicators are used, such as color-change and melting indicators, to give visual indication that a package or truckload has been subjected to a sterilization process. Such evidence can become a part of the batch record to confirm that sterilization was accomplished.

Further details concerning methods of sterilization and their application will be found in Chapter 84. In addition, the USP provides suggestions concerning the sterilization of injections and related materials.

Freeze-Drying

Freeze-drying (lyophilization) is a process of drying in which water is sublimed from the product after it is frozen.²⁴ The particular advantages of this process are that biologicals and pharmaceuticals which are relatively unstable in aqueous solution can be processed and filled into dosage containers in the liquid state, taking advantage of the relative ease of processing a liquid. They can be dried without elevated temperatures, thereby eliminating adverse thermal effects, and stored in the dry state in which there are relatively few stability problems.

Further advantages are that these products are often more soluble and/or more rapidly soluble, dispersions are stabilized throughout their shelf life and products subject to degradation by oxidation have enhanced stability because the process is carried out in a vacuum.

However, the increased time and handling required for processing and the cost of the equipment limit the use of this process to those products which significantly have enhanced stability if stored in the dry state.

The fact that ice will sublime at pressures below 3 torr has been a long-established laboratory principle (see Chapter 19). The extensive program for freeze-drying human plasma during World War II provided the impetus for the rapid development of the process.

Freeze-drying essentially consists of

1. Freezing an aqueous product at a temperature below its eutectic temperature.
2. Evacuating the chamber, usually below 0.1 torr (100 μ m Hg).
3. Subliming ice on a cold condensing surface at a temperature below that of the product, the condensing surface being within the chamber or in a connecting chamber.
4. Introducing heat to the product under controlled conditions, thereby providing energy for sublimation at a rate designed to keep the product temperature below its eutectic temperature.

Figure 26 shows such a system. The product may be fro-

R
O
/

zen
(usu
sor t
plet
conc
surfa
ant f
Hu
elec
glyc
ally
solid
cont
F
uct
com
ing
fore
fer l
the
unti
T
diag
ted
ber
ser
the
It
am
duc
atr
mo
ver
mu
I
dep
dry
the
fill
tio
the
wh
liqu

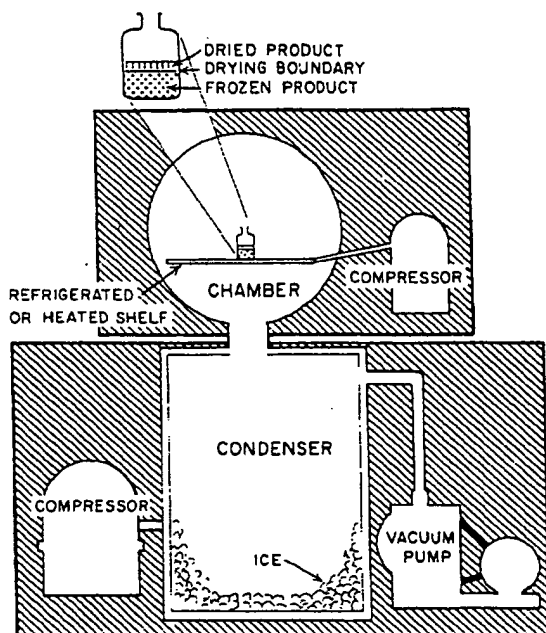


Fig 26. Essential components of a freeze-drying system.

zen on the shelf in the chamber by circulating refrigerant (usually Freon, ammonia or ethylene glycol) from the compressor through pipes within the shelf. After freezing is complete, which may require several hours, the chamber and condenser are evacuated by the vacuum pump, the condenser surface having been chilled previously by circulating refrigerant from the large compressor.

Heat then is introduced from the shelf to the product by electric resistance coils or by circulating hot water, silicone or glycol. The process continues until the product is dry (usually 1% or less moisture), leaving a sponge-like matrix of the solids originally present in the product, the input of heat being controlled so as not to degrade the product.

For most pharmaceuticals and biologicals the liquid product is sterilized by filtration and then filled into the dosage container aseptically. The containers must remain open during the drying process to allow water vapor to escape; therefore, they must be protected from contamination during transfer from the filling area to the freeze-drying chamber, while in the freeze-drying chamber and at the end of the drying process until sealed.

The chambers may be equipped with hydraulic or rubber diaphragm internal-stoppering devices designed to push slotted rubber closures into the vials to be sealed while the chamber is still evacuated, the closures having been partially inserted immediately after filling so that the slots were open to the outside.

If internal stoppering is not available or containers such as ampuls are used, filtered dry air or nitrogen must be introduced to the chamber at the end of the process to establish atmospheric pressure. Then the containers must be removed and sealed under aseptic conditions. If the product is very sensitive to moisture, the environmental humidity also must be controlled until it is sealed.

Factors Affecting the Process Rate—The greater the depth of the product in the container, the longer will be the drying process. Therefore, a product to be frozen by placing the container on a refrigerated shelf (plug freezing) should be filled to a planned, limited depth. If a large volume of solution must be processed, the surface area may be increased and the depth decreased by freezing the solution on a slant or while rotating the container on an angle (shell freezing) in a liquid refrigerant bath, such as dry ice and alcohol.

The actual driving force for the process is the vapor pressure differential between the vapor at the surface where drying of the product is occurring (the drying boundary) and that at the surface of the ice on the condenser. The latter is determined by the temperature of the condenser as modified by the insulating effect of the accumulated ice. The former is determined by a number of factors, including:

1. The rate of heat conduction through the container and the frozen material, both usually relatively poor thermal conductors, to the drying boundary while maintaining all of the product below its eutectic temperature.
2. The impeding effect of the increasing depth of dried, porous product above the drying boundary.
3. The temperature and heat capacity of the shelf itself.

This may be visualized by referring to Fig 26.

The passageways between the product surface and the condenser surface must be wide open and direct for effective operation. Therefore, the condensing surfaces in large freeze-driers are usually in the same chamber as the product. Evacuation of the system is necessary to reduce the impeding effect that collisions with air molecules would have on the passage of water molecules. However, the residual pressure in the system must be greater than the vapor pressure of the ice on the condenser or the ice will be vaporized and pulled into the pump, an event detrimental to most pumps.

The amount of solids in the product, their particle size and their thermal conductance will affect the rate of drying. The more solids present, the more impediment will be provided to the escape of the water vapor. The smaller the particle size, particularly the crystal size of the ice, the faster the drying generally will be. The poorer the thermal conducting properties of the solids in the product, the slower will be the rate of heat transfer through the frozen material to the drying boundary.

The rate of drying is essentially slow, most often requiring 24 hours or longer for completion. The actual time required, the rate of heat input and the product temperatures that may be used must be determined for each product and then reproduced carefully with successive processes.

Factors Affecting Formulation—The active constituent of many pharmaceutical products is present in such a small quantity that if freeze-dried alone its presence would be hard to detect visually. Therefore, excipients often are added to increase the amount of solids.

Some consider it ideal for the dried-product plug to occupy essentially the same volume as that of the original solution. To achieve this, the solids content of the original product must be between approximately 5 and 25%. Among the substances found most useful for this purpose, usually as a combination, are sodium or potassium phosphates, citric acid, tartaric acid, gelatin and carbohydrates such as dextrose, mannitol and dextran.

Each of these substances contributes appearance characteristics of the plug, such as whether dull and spongy or sparkling and crystalline, firm or friable, expanded or shrunken and uniform or striated. Therefore, the formulation of a product to be freeze-dried must include consideration not only of the nature and stability characteristics required during the liquid state, both freshly prepared and when reconstituted before use, but the characteristics desired in the dried plug.

Modifications in the Process and Equipment—In some instances a product may be frozen in a bulk container or in trays rather than in the final container and then handled as a dry solid. This may be desirable when large volumes of a product are processed, but the risk of contamination from the environment is high.

When large quantities of material are processed it may be desirable to use ejection pumps in the equipment system. These draw the vapor into the pump and eject it to the outside, thereby eliminating the need for a condensing surface. Such pumps are expensive and usually practical only in large installations.



Fig 27. Aseptic loading of freeze-drier (courtesy, Upjohn).

Available freeze-driers (Suppliers: *Edwards, Finn-Aqua, ETS, Hull, NRC, Stokes, Virtis*) range in size from small laboratory units to large industrial models such as the one shown in Fig 27. Their selection requires consideration of such factors as

- The tray area required.
- The volume of water to be removed.
- Whether or not aseptic processing will be involved.
- Is internal stoppering required?
- Will separate freezers be used for initial freezing of the product.
- The degree of automatic operation desired.

Other factors involved in the selection and use of equipment are considered in the literature.²⁵

Freeze-drying is now being used for research in the preservation of human tissue and is finding increasing application in the food industry. Progress on new developments is being made in both the process and the equipment.²⁶

Quality Assurance and Control

The importance of undertaking every possible means to assure the quality of the finished product cannot be overemphasized. Every component and step of the manufacturing process must be subjected to intense scrutiny to be confident that quality is attained in the finished product. The responsibility for achieving this quality is divided appropriately in concept and practice into Quality Assurance (QA) and Quality Control (QC). QA relates to the studies made and the plans developed for assuring quality of a product prospectively. QC embodies the carrying out of these plans during production and includes all of the tests and evaluations performed to be sure that quality has been achieved in a specific lot of product.

The principles for achieving quality are basically the same for the manufacture of any pharmaceutical. These are discussed in Chapter 39. During the discussion of the preparation of injections, mention was made of numerous quality requirements for components and manufacturing processes. Here, only selected tests characteristically required before a finished product is released will be discussed briefly, including sterility, pyrogen and particulate tests.

Sterility Test

All lots of injections in their final containers must be tested for sterility. The USP prescribes the requirements for this test for official injections. The FDA uses these requirements as a guide for testing unofficial sterile products. The official test has acknowledged limitations in the information that it can provide, particularly those limitations inherent in microbiological procedures. Therefore, it should be noted that this test is not intended as a thoroughly evaluative test for a product subjected to a sterilization method of unknown effectiveness. It is intended primarily as a check test on the probability that a previously validated sterilization procedure has been repeated, or to give assurance of its continued effectiveness. A discussion of sterility testing is given in Chapter 84.

In the event of a sterility-test failure, the immediate issue concerns whether the growth observed came from viable microorganisms in the product (true contamination) or from adventitious contamination during the testing (a false positive). The USP does permit a retest but the position of the FDA is that retest results are only valid if persuasive evidence exists that the cause of the initial sterility-test failure resides in the laboratory. Therefore, a thorough investigation must be

launched to support the justification for performing the retest and assessing the validity of the retest results relative to release of the lot of product.

It should be noted that a "lot" with respect to sterility testing is that group of product containers which has been subjected to the same sterilization procedure. For containers of a product which have been sterilized by autoclaving, for example, a lot would constitute those processed in a particular sterilizer cycle. For an aseptic filling operation, a lot would constitute all of those product containers filled during a period when there was no change in the filling assembly or equipment and which is no longer than one working day or shift.

Pyrogen Test

The USP evaluates the presence of pyrogens in parenteral preparations by a qualitative fever response test in rabbits, the Pyrogen Test (Section (151)), and by the Bacterial Endotoxins Test (Section (85)). These two USP tests are described in Chapter 30. Rabbits are used as test animals in Section (151) because they show a physiological response to pyrogenic substances similar to that by man. While a minimum pyrogenic dose (MPD), the amount just sufficient to cause a positive USP Pyrogen Test response, sometimes may produce uncertain test results, a content equal to a few times the MPD will leave no uncertainty. Therefore, the test is valid and has continued in use since introduced by Seibert in 1923. It should be understood that not all injections may be subjected to the rabbit test since the medicinal agent may have a physiological effect on the test animal such that any fever response would be masked.

The *Bacterial Endotoxins Test* is an *in vitro* test based on the formation of a gel or the development of color in the presence of bacterial endotoxins and the lysate of the amoebocytes of the horseshoe crab (*Limulus polyphemus*). The *Limulus Amoebocyte Lysate* (LAL) test, as it also is called, is a biochemical test performed in a test tube, and is simpler, more rapid and of greater sensitivity than the rabbit test.²⁷ Although it detects only the endotoxic pyrogens of gram-negative bacteria, these are the most prominent environmental microbial contaminants likely to invade sterile products. The test also has been automated.²⁸

The LAL test is a semiquantitative test. To provide standardization for the test, the USP has established a reference endotoxin against which lots of the lysate are standardized.

Thus, the sensitivity of the lysate is given in terms of endotoxin units (EU). Most USP injections now have been given limits in terms of EUs (eg, Bacteriostatic Sodium Chloride Injection, 1.0 EU/mL) as another measure of the purity of the product.

Particulate Evaluation

Particulate matter in parenteral solutions long has been recognized as unacceptable since the user could be expected to conclude that the presence of visible "dirt" would suggest that the product is of inferior quality. Today, it is recognized that the presence of particles in solution, particularly if injected intravenously, can be harmful. While data defining the extent of risk and the effects produced still are limited, it has been shown that particles of lint, rubber, insoluble chemicals and other foreign matter can produce emboli in the vital organs of animals and man.²⁹ Further, it has been shown that the development of infusion-phlebitis may be related to the presence of particulate matter in intravenous fluids.³⁰

The particle size of particular concern has not been clearly delineated, but it has been suggested that since erythrocytes have a diameter of approximately 4.5 μm , particles of more than 5 μm should be the basis for evaluation. This is a considerably smaller particle than can be seen with the unaided eye; approximately 50 μm is the lower limit unless the Tyndall effect is used whereby particles as small as 10 μm can be seen by the light scattered from them.

The USP specifies that good manufacturing practice requires that each final container of an injection be subjected individually to a visual inspection and that containers in which visible particles can be seen should be discarded. This 100% inspection of a lot of product is designed to prevent the distribution and use of parenterals which contain particulate matter that may be harmful psychologically or organically to the participant. Therefore, all of the product units from a production line currently are being inspected individually by human inspectors under a good light, baffled against reflection into the eye and against a black-and-white background. This inspection is subject to the limitation of the size of particles that can be seen, the variation of visual acuity from inspector to inspector, their emotional state, eye strain, fatigue and other personal factors that will affect what is seen. However, it does provide a means for eliminating the few units which normally contain visible particles. Slow progress has been made on developing equipment for automated inspection of product containers.

Since it is recognized that visual inspection will not detect the presence of particles smaller than approximately 50 μm in size, the USP has established a microscopic test method for identifying particles in large-volume intravenous solutions and has set limits of not more than 50 particles/mL of 10 μm and larger in size and not more than 5 particles/mL of 25 μm and larger in size. This method consists essentially of filtering a measured sample of solution through a membrane filter under ultraclean conditions and then counting the particles on the surface of the filter using oblique light, under a microscope, at both 40x and 100x magnification. These standards are being met readily by the large-volume parenteral solutions currently being manufactured in the US.

More recently the USP established standards for small-volume parenterals to be given intravenously, using an electronic instrument that counts and measures the size of particles by means of a shadow cast by the particle as it passes through a high-intensity light beam (Suppliers: *Climet*, *HLAC*). The limits prescribed are not more than 10,000 particles/container of $\geq 10 \mu\text{m}$ in size and not more than 1000 particles/container $\geq 25 \mu\text{m}$ in size. These specifications were developed on the premise that as many as five such products may be added to a 1-L bottle of a large-volume parenteral and five products should not contribute more than the overall limits of particles prescribed for a large-volume

parenteral. Whether or not these standards are realistic toxicologically has not been established; rather, the objective of the compendium is to establish specification limits that would encourage the preparation of clean parenteral solutions, particularly for those to be given intravenously.

It also should be realized that administration sets and the techniques used in the hospital for preparing and administering intravenous infusion fluid may introduce substantial amounts of particulate matter into an otherwise clean solution. Therefore, the pharmaceutical manufacturer, the administration set manufacturer, the hospital pharmacist, the nurse and the physician must share responsibilities for making sure that the patient receives a clean intravenous injection.

The USP methods for counting and sizing particulate matter in intravenous solutions are not the only methods available for such determinations. A number of electronic particle counters are available that use the light-scattering principle to count particles in a liquid sample (Suppliers: *Climet*, *Mei One*, *HLAC/Royco*). There also is an instrument available which counts particles and sizes them by measuring the effect on the resistance between two electrodes as the particles pass between them (Supplier: *Coulter*). It is obvious that only the visual inspection can be used for in-line evaluation of every container produced commercially. All of these methods require very stringent ultraclean preparation techniques to assure reasonable accuracy in counting and sizing only the particles in the solution, rather than those that may have been introduced inadvertently during the sample preparation or the testing procedure. Further, these test procedures are destructive and, therefore, can be performed only on samples of the production lot. Further information may be found in a review article.³¹

Leaker Test

Ampuls that have been sealed by fusion must be subjected to a test to determine whether or not a passageway remains to the outside; if so, all or a part of the contents may leak to the outside and spoil the package, or microorganisms or other contaminants may enter. Changes in temperature during storage cause expansion and contraction of the ampul and contents, and will accentuate interchange if a passageway exists, even if microscopic in size.

This test usually is performed by producing a negative pressure within an incompletely sealed ampul while the ampul is entirely submerged in a deeply colored dye solution. Most often, approximately 1% methylene blue solution is employed. The test may be performed by subjecting the ampuls to a vacuum in a vacuum chamber, the ampuls being submerged in a dye bath throughout the process. Another procedure frequently employed is to simply autoclave the ampuls in a dye bath. A modification of this is to remove them from the autoclave while hot and quickly submerge them in a cool bath of dye solution. After carefully rinsing the dye solution from the outside, color from the dye will be visible within a leaker. Leakers, of course, are discarded.

Vials and bottles are not subjected to a leaker test because the sealing material (rubber stopper) is not rigid. Therefore, results from such a test would be meaningless. However, evacuated bottles containing a liquid may be checked for a sharp "click" sound produced when struck with an implement such as a rubber mallet or the ball of the hand. However, assurance of container-closure sealing integrity should be an integral part of product development by developing specifications for the fit of the closure in the neck of the container, the physical characteristics of the closure, the need for lubrication of the closure and the capping pressure.

Safety Test

The National Institutes of Health requires of most biological products routine safety testing in animals. Under the Kefauver-Harris Amendments to the Federal Food, Drug, and Cos-

metic Act, most pharmaceutical preparations are now required to be tested for safety. Because it is entirely possible for a parenteral product to pass the routine sterility test, pyrogen test and chemical analyses and still cause unfavor-

able reactions when injected, a safety test in animals is essential to provide additional assurance that the product does not have unexpected toxic properties. Safety tests in animals are discussed in detail in the USP.

Packaging and Labeling

A full discussion of the packaging of parenteral preparations is beyond the scope of this text. It is essential, of course, that the packaging should provide ample protection for the product against physical damage from shipping, handling and storage as well as protecting light-sensitive materials from ultraviolet radiation. An extensive review of this subject has been published.³²

Packaging—The USP includes certain requirements for the packaging and storage of injections, as follows:

1. The volume of injection in single-dose containers is defined as that which is specified for parenteral administration at one time and is limited to a volume of 1 L.
2. Parenterals intended for intraspinal, intracisternal or peridural administration are packaged only in single-dose containers.
3. Unless an individual monograph specifies otherwise, no multiple-dose container shall contain a volume of injection more than sufficient to permit the withdrawal and administration of 30 mL.
4. Injections packaged for use as irrigation solutions or for hemofiltration or dialysis or for parenteral nutrition are exempt from the foregoing requirements relating to packaging. Containers for injections packaged for use as hemofiltration or irrigation solutions may be designed to empty rapidly and may contain a volume in excess of 1 L.
5. Injections intended for veterinary use are exempt from the packaging and storage requirements concerning the limitation to single-dose containers and to volume of multiple-dose containers.

Labeling—The labeling of an injection must provide the physician or other user with all of the information needed to assure the safe and proper use of the therapeutic agent. Since all of this information cannot be placed on the immediate container and be legible, it may be provided on accompanying printed matter. General labeling requirements for drugs are discussed in Chapter 110.

A restatement of the labeling definitions and requirements of the USP for Injections is as follows:

The term "labeling" designates all labels and other written, printed or graphic matter upon an immediate container or upon, or in, any package or wrapper in which it is enclosed, with the exception of the outer shipping container. The term "label" designates that part of the labeling upon the immediate container.

The label states the name of the preparation, the percentage content of drug of a liquid preparation, the amount of active ingredient of a dry preparation, the volume of liquid to be added to prepare an injection or suspension from a dry preparation, the route of administration, a statement of storage conditions and an expiration date. Also, the label must indicate the name of the manufacturer or distributor and carry an identifying lot number. The lot number is capable of providing access to the complete manufacturing history of the specific package, including each single manufacturing step.

The container label is so arranged that a sufficient area of the container remains uncovered for its full length or circumference to permit inspection of the contents.

The label must state the name of the vehicle and the proportions of each constituent, if it is a mixture; the names and proportions of all substances added to increase stability or usefulness and the expiration date where required by the individual monograph.

Preparations labeled for use as dialysis, hemofiltration or irrigation solutions must meet the requirements for injections other than those relating to volume and also must bear on the label statements that they are not intended for intravenous injection.

Injections intended for veterinary use are so labeled.

References

1. Griffenhagen GB: *Bull Parenter Drug Assoc* 16(2): 12, 1962.
2. Joint Commission on Accreditation of Healthcare Organizations, Accreditation Manual for Hospitals, p 107, Chicago, 1992.
3. Trissel LA: *Handbook on Injectable Drugs*, 7th ed, Am Soc Hosp Pharm, Bethesda, MD, 1992.
4. 21 CFR 210, Current Good Manufacturing Practice in manufacturing, processing, packaging or holding of drugs; general, Supt. of Documents, USGPO, Washington DC.
5. *Tech Rep No 4*, Parenteral Drug Association (PDA), Philadelphia, 1983.
6. Brown J et al: *Pharm Eng* 11(4): 15, 1991.
7. Spiegel AJ, Noseworthy MM: *J Pharm Sci* 52: 917, 1963.
8. Schroeter LC: *Ibid* 50: 891, 1961.
9. Pearson FC III: *Pyrogens*, Dekker, New York, 1985.
10. Morton DK: *J Parenter Sci Technol* 41: 145, 1987.
11. Autian J: *Bull Parenter Drug Assoc* 22: 276, 1968.
12. Lambert P: *Pharm Technol* 15: 48, 1991.
13. *Tech Methods Bull No 3*, PDA, Philadelphia, 1982.
14. *Tech Methods Bull No 1*, PDA, Philadelphia, 1980.
15. *Tech Methods Bull No 2*, PDA, Philadelphia, 1981.
16. Fed Sid No 209E, GSA, Washington, DC 20407, Sep. 11, 1992.
17. Davenport SM: *Proc PharmTech Conf*, 92, 1992.
18. Whyte W, Niven L: *J Parenter Sci Technol* 40: 182, 1986.
19. Grimes TL, Fonner DE et al: *Ibid* 31: 179, 1977.
20. Myers T, Chrai S: *J Parenter Sci Technol* 35: 8, 1981.
21. Seiberling DA: *Pharm Eng* 6(6): 30, 1986.
22. Singh M, Ravin LJ: *Ibid* 40: 34, 1986.
23. Levy RV, Souza KS, Neville CB: *Pharm Technol* 14: 160, 1990.
24. *Ann NY Acad Sci* 85: 501-734, 1965.
25. Morgan SL, Spotts MR: *Pharm Technol* 3: 94-101, 114, 1979.
26. Nail SL, Gatlin LA: *J Parenter Sci Technol* 39: 16, 1985.
27. Cooper JF: *Bull Parenter Drug Assoc* 29: 122, 1975.
28. Novitsky TJ, Ryther SS et al: *J Parenter Sci Technol* 36: 11, 1982.
29. Garvan JM, Gunner BW: *Med J Aust* 2: 1, July 4, 1964.
30. Deluca P et al: *Am J Hosp Pharm* 32: 1001, 1975.
31. Borchert SJ, Abe A et al: *J Parenter Sci Technol* 40: 212, 1986.
32. *Tech Report No. 5*, PDA, Philadelphia, 1984.

Bibliography

- Akers MJ: *Parenteral Quality Control*, Dekker, New York, 1985.
- Avis KE, Levchuk JW. In King RE, ed: *Dispensing of Medication*, 9th ed, Mack Publ Co. Easton PA, Chap 9, 1984.
- Avis KE, Lieberman HA, Lachman L, eds: *Pharmaceutical Dosage Forms: Parenteral Medications*, 2nd ed, vol 1, Dekker, New York, 1992.
- Ibid*, 2nd ed, vol 2, Dekker, New York, 1993.
- Ibid*, 2nd ed, vol 3, Dekker, New York, 1993.
- Avis KE. In Lachman L et al: *The Theory and Practice of Industrial Pharmacy*, 3rd ed, Lea & Febiger, Philadelphia, Chaps 21 & 22, 1986.
- Block SS, ed: *Disinfection, Sterilization and Preservation*, 3rd ed, Lea & Febiger, Philadelphia, 1983.
- Carleton FJ, Agalloco JP, eds: *Validation of Aseptic Pharmaceutical Processes*, Dekker, New York, 1986.
- Gaughran ERL, Kereluk K, eds: *Sterilization of Medical Products*, Johnson & Johnson, New Brunswick NJ, 1977.
- Martin EW et al: *Techniques of Medication*, Lippincott, Philadelphia, 1969.
- Meltzer TH, ed: *Filtration in the Pharmaceutical Industry*, Dekker, New York, 1987.
- Meryman HT, ed: *Cryobiology*, Academic, New York, 1966.
- Pearson FC III: *Pyrogens*, Dekker, New York, 1985.
- Phillips GB, Miller WS, eds: *Industrial Sterilization*, Duke Univ Press, Durham NC, 1973.
- Turco S, King RE: *Sterile Dosage Forms*, 3rd ed, Lea & Febiger, Philadelphia, 1987.

CHAPTER 88

Intravenous Admixtures

Salvatore J Turco, PharmD, FASHP

Professor of Pharmacy
Temple University School of Pharmacy
Philadelphia, PA 19140

△ NOTICE: THIS MATERIAL MAY BE PROTECTED
BY COPYRIGHT LAW (TITLE 17 U.S. CODE)

It has been estimated that 40% of all drugs administered in hospitals are given in the form of injections and their use is increasing. Part of this increase in parenteral therapy is due to the wider use of intravenous fluids (IV fluids). In the last decade the use of IV fluids has doubled, increasing from 150 million units to 320 million units annually. Not only do IV fluids continue to serve as the means for fluid replacement, electrolyte-balance restoration and supplementary nutrition, but they also are playing major roles as vehicles for administration of other drug substances and in total parenteral nutrition (TPN). Intravenous fluids are finding greater use as the means of administering other drugs because of convenience, the means of reducing the irritation potential of the drugs and the desirability for continuous and intermittent drug therapy. The techniques for providing TPN parenterally have improved steadily in the last decade, and such use is increasing markedly. The use of IV fluids for these purposes requires the compounding of specific intravenous admixtures (parenteral prescriptions) to meet the clinical needs of a given patient. However, the combination of drug substances in an IV fluid can promote parenteral incompatibilities and give rise to conditions not favorable for drug stability. A new area of specialization has been created for hospital pharmacists who can develop the expertise to prepare these solutions—recognizing their compatibility and stability problems and the potential for contamination—and participate in the administration of the solutions. The complex compounding of an order for TPN requires knowledgeable personnel capable of making accurate calculations, compounding and having aseptic technique. The parenteral prescription is becoming increasingly important in hospitals. Centralized admixture programs are now found in 85% of the nation's hospitals having 300 beds or more. Equipment available for administering IV fluids has become more sophisticated, and has made possible increased accuracy of dosage and led to the development of new concepts and methods of nutrition and drug therapy.

Electronic mechanical equipment is now commonplace in hospitals. Its use, as well as its sophistication, continues to increase. Newly designed electronic pumps have been developed for hospital ambulatory use. Multichannel pumps have become available for multiple-drug infusion. Over 500,000 implantable infusion ports have been inserted into patients and 100,000 new patients receive these implantable ports each year in order to accomplish drug therapy. New methods of IV drug delivery systems have been introduced and are constantly evolving. The introduction of patient controlled analgesia (PCA) is commonplace in hospitals. This technology allows the patient with pain to control the degree of analgesia desired.

The growth of TPN in hospitals has been paralleled by home TPN programs. Large numbers of patients conduct parenteral nutrition in the home environment, including those with infectious and neoplastic diseases. More stringent and complete guidelines for the preparation and parenterals in hospitals by pharmacists have been published. These guidelines, promoting sophisticated methods of preparation by the pharmacist, have become recommendations. They are a testament to the importance of parenteral preparation in the institutional setting. Packaging of parenterals in the past five

years also has undergone dramatic changes. Prefilled, premixed, prefrozen parenterals are now supplied by the manufacturers. Newly designed plastic mini-bags (ADD-Vantage Abbott) have been introduced. Premixed liquids (eg, antibiotics, theophylline, heparin, lidocaine, dopamine) are available from parenteral manufacturers. Multiple-dose containers (Marivials, Lyphomed) have been developed to accommodate new methods of preparation of parenterals by the pharmacist. The pharmaceutical industry has responded to the needs of pharmacists by addressing the packaging, labeling and design requirements necessary to facilitate patient care. The parenteral drug industry continues its efforts to meet higher standards of quality and to assure the availability of sterile and particulate-free products.

Intravenous Fluids

Large-volume injections intended to be administered by intravenous infusion commonly are called IV fluids and are included in the group of sterile products referred to as large-volume parenterals. These consist of single-dose injections having a volume of 100 mL or more and containing no added substances. Intravenous fluids are packaged in containers having a capacity of 100 to 1000 mL. Minitype infusion containers of 250-mL capacity are available with 50- and 100-mL partial fills for solution of drugs when used in the "piggyback" technique (ie, the administration of a second solution through a Y-tube or gum-rubber connection in the administration set of the first intravenous fluid, thus avoiding the need for another injection site). In addition to the IV fluids, this group also includes irrigation solutions and solutions for dialysis.

Intravenous fluids are sterile solutions of simple chemicals such as sugars, amino acids or electrolytes—materials which easily can be carried by the circulatory system and assimilated. Prepared with Water for Injection USP, the solutions are pyrogen-free. Because of the large volumes administered intravenously, the absence of particulate matter assumes a significant role in view of possible biological hazards resulting from insoluble particles. Absence of particulate matter or clarity of IV fluids is as important at the time of administration following their manipulation in the hospital as it is at the time of manufacture of the injection.

Limits for particulate matter occurring in IV fluids, or large-volume injections used for single-dose infusion, are defined in the USP. This represents the first regulatory attempt to define limits for particulate matter in parenterals. Limits also apply to multiple-dose injections, small-volume injections or injections prepared by reconstitution from sterile solids. The USP defines particulate matter as extraneous, mobile, undissolved substances, other than gas bubbles, unintentionally present in parenteral solutions. The total numbers of particles having effective linear dimensions equal to or larger than 10 μm and larger than 25 μm are counted. The IV fluid meets the requirement of the test if it contains not more than 50 particles per mL which are equal to or larger than 10 μm , and not more than 5 particles per mL which are equal to or larger than 25 μm in linear dimension.

Intravenous fluids commonly are used for a number of clinical conditions. These include

- Correction of disturbances in electrolyte balance.
- Correction of disturbances in body fluids (fluid replacement).
- The means of providing basic nutrition.
- The basis for the practice of providing TPN.
- Use as vehicles for other drug substances.

In both of the latter two cases it has become common practice to add other drugs to certain IV fluids to meet the clinical needs of the patient. Using IV fluids as vehicles offers the advantages of convenience, the means of reducing the irritation potential of the drug and a method for continuous drug therapy. However, the practice requires that careful consideration be given to the stability and compatibility of additives present in the IV fluids serving as the vehicle. This approach also demands strict adherence to aseptic techniques in adding the drugs, as well as in the administration of the IV fluids. These procedures are discussed later in the chapter. The IV fluids commonly used for parenterals are shown in Table 1.

Many disease states result in electrolyte depletion and loss. Proper electrolyte concentration and balance in plasma and tissues are critical for proper body function. Electrolyte restoration and balance are achieved most rapidly through administration of IV fluids. Required electrolytes include sodium and chloride ions, which in normal saline more closely approxi-

mate the composition of the extracellular fluid than solutions of any other single salt; potassium, the principal intracellular cation of most body tissues and essential for the functioning of the nervous and muscular systems as well as the heart; magnesium, as a nutritional supplement especially in TPN solutions and phosphate ion, important in a variety of biochemical reactions. In addition to the number of standard electrolyte fluids shown in Table 1, a large number of combinations of electrolytes in varying concentrations are available commercially. Some of these electrolyte fluids also contain dextrose.

Dextrose Injection 5% (D5/W) is the most frequently used IV fluid, either for nutrition or fluid replacement. It is isotonic and administered intravenously into a peripheral vein; 1 g of dextrose provides 3.4 cal and 1 L of D5/W supplies 170 cal. The body uses dextrose at a rate of 0.5 g per kg of body weight per hour. More rapid administration can result in glycosuria. Therefore, 1 L of D5/W requires 1½ hours for assimilation. The pH range of D5/W can vary from 3.5 to 6.5. The wide range permitted is due to the free sugar acids present and formed during the sterilization and storage of the injection. To avoid incompatibilities when other drug substances are added to Dextrose Injection, the possible low pH should be considered in using it as a vehicle. More concentrated solutions of dextrose are available and provide in-

Table 1—Fluids Used Commonly for IV Use

Injection	Concentration (%)	pH	Therapeutic use
Alcohol			
with D5/W ^a	5	4.5	Sedative, analgesic, calories
with D5/W in NSS ^b	5		Sedative, analgesic, calories
Amino acid (synthetic)			Fluid and nutrient replenisher
Aminosyn II (Abbott)	3.5; 7	5.25	
FreAmine III (McGraw)	8.5	6.6	
Travasol (Baxter)	3.5; 5.5; 8.5	6.0	
Ammonium chloride	2.14	4.5–6.0	Metabolic alkaloids
Dextran 40			
in NSS	10	5	Priming fluid for extracorporeal circulation
in D5/W	10	4	Priming fluid for extracorporeal circulation
Dextran 70			
in NSS	6	5	Plasma volume expander
in D5/W	6	4	Plasma volume expander
Dextrose (glucose, D5/W)	2.5–50	3.5–6.5	Fluid and nutrient replenisher
Dextrose and sodium chloride	Varying concn of dextrose from 5–20 with varying concn of sodium chloride from 0.22–0.9	3.5–6.5	Fluid, nutrient and electrolyte replenisher
Invert sugar (fructose and dextrose)	5; 10	4.0	Fluid and nutrient replenisher
Lactated Ringer's (Hartmann's)		6.0–7.5	Systemic alkalinizer; fluid and electrolyte replenisher
NaCl	0.6		
KCl	0.03		
CaCl ₂	0.02		
Lactate	0.3		
Mannitol	5	5.0–7.0	Osmotic diuresis
also in combination with dextrose or sodium chloride	10 15 20		
Multiple electrolyte solutions varying combinations of electrolytes, dextrose, fructose, invert sugar		5.5	Fluid and electrolyte replacement
Ringer's		5.0–7.5	Fluid and electrolyte replenisher
NaCl	0.86		
KCl	0.03		
CaCl ₂	0.033		
Sodium bicarbonate	5	8	Metabolic acidosis
Sodium chloride	0.45; 0.9; 3; 5	4.5–7.0	Fluid and electrolyte replenisher
Sodium lactate	1/6 M	6.3–7.3	Fluid and electrolyte replenisher
Sterile water for injection		5.5	Diluent

^a5% Dextrose in water.

^bNormal saline solution.

creas
tonic
perip
tered
nous
tainu
unlik
injec
suga
clair
utiliz
Intr
prov
(Cha
repa
for tl
unde
mair
tein
down
whic
bala
elim
nitro
bein
are
takir
requ
(dis
peri
thes
lyte
Vitr
som
ano
pac
fat i

C
tain
ter)
stor
sur
rior
reg
abl
fro
are
tio
mL
use
inj
vac
lea
tio
the
qu
sw
be
flu
for
lat
tai
pe
an
nu
ey
Be
co
Ga
sy

creased calorie intake with less fluid volume. Being hypertonic, the more concentrated solutions may be irritating to peripheral veins. Highly concentrated solutions are administered in a larger central vein. Other IV fluids used for intravenous admixtures and providing calories include solutions containing invert sugar. There is some evidence that fructose, unlike dextrose, may be used in diabetic patients; the 10% injection is hypertonic and provides 375 cal per L. Invert sugar consists of equal parts of dextrose and fructose; it is claimed that the presence of fructose promotes more rapid utilization of dextrose.

Intravenous fluids containing crystalline amino acids can provide biologically usable amino acids for protein synthesis (Chapter 65). Protein contributes to tissue growth, wound repair and resistance to infection. The protein requirement for the normal adult is 1 g per kg per day; children and patients under stress require greater amounts. Attempts are made to maintain a positive nitrogen balance, indicating that the protein administered is being utilized properly and not broken down and eliminated through the urine as creatinine and urea, which are normal waste products. In a positive nitrogen balance patients are taking in more nitrogen than they are eliminating. In a negative nitrogen balance there is more nitrogen being eliminated through the urine regularly than is being administered intravenously. This means that tissues are continuing to be torn down and repair is not necessarily taking place. Amino Acid Injection can afford the total body requirements for proteins by the procedure known as TPN (discussed below) or be used for supplemental nutrition by peripheral administration. In addition to the amino acids, these nutritional injections also may contain dextrose, electrolytes, vitamins and insulin. Fat emulsion (*Intralipid*, Kabi Vitrum AB; *Liposyn II*, Abbott and *Travamulsion*, Baxter) sometimes is used concurrently but usually administered at another site. However, new systems such as three-in-one packaging permit mixing of amino acids, carbohydrates and fat in one container for TPN.

Packaging Systems

Containers for intravenous fluids must be designed to maintain solution sterility, clarity (freedom from particulate matter) and nonpyrogenicity from the time of preparation, through storage and during clinical administration. Container closures must be designed to facilitate insertion of administration sets through which the injections are administered, at a regulated flow-rate, into suitable veins. IV fluids are available in glass and plastic containers; the latter may be made from either a flexible or semirigid plastic material. IV fluids are supplied in 1000-mL, 500-mL and 250-mL sizes in addition to 250-mL capacity containers packaged with 50 or 100 mL of D5/W or sodium chloride injection 0.9% for piggyback use in addition to 0.45% sodium chloride and 2.5% dextrose injections. IV fluids in glass containers are packaged under vacuum, which must be dissipated prior to use. For fluid to leave the IV glass container and flow through the administration set, some mechanism is necessary to permit air to enter the container. Current flexible plastic systems do not require air introduction in order to function. Atmospheric pressure pressing on the container forces the fluid to flow.

All glass and plastic containers are single-dose and should be discarded after opening even if not used. Intravenous fluids are packaged with approximately 3% excess fill to allow for removal of air from the administration set and permit the labeled volume to be delivered from the container. The containers are graduated at 20-mL increments on scales that permit the volume in container to be determined either from an upright or inverted position. Glass containers have aluminum and plastic bands for hanging, while plastic containers have eyelet openings or plastic straps for attachment to IV poles.

Fluids for IV use are available from three sources (Abbott, Baxter and McGaw); all provide both glass and plastic containers. The glass-container systems of Baxter and McGaw are similar. The characteristics of current packaging systems are summarized in Table 2.

Table 2—IV Fluid Systems

Source	Container	Characteristics
Baxter	Glass	Vacuum Air tube
Baxter (<i>Viaflex</i>)	Plastic	Polyvinyl chloride Flexible Nonvented
McGaw	Glass	Vacuum Air tube
McGaw (<i>Excell</i>)	Plastic	Flexible
McGaw (<i>Accumed</i>)	Plastic	Polyolefin Semirigid Vacuum
Abbott	Glass	Air filter ^a
Abbott (<i>Lifecare</i>)	Plastic	Polyvinyl chloride Flexible Nonvented

^aPart of administration set.

Administration Sets

Administration sets used to deliver fluids intravenously are sterile, pyrogen-free and disposable. Although these sets are supplied by different manufacturers, each for its own system, they have certain basic components. These usually include a plastic spike to pierce the rubber closure or plastic seal on the IV container, a drip (sight) chamber to trap air and permit adjustment of flow rate and a length (150 to 450 cm) of polyvinyl chloride tubing terminating in a gum-rubber injection port. At the tip of the port is a rigid needle or catheter adapter. An adjustable clamp (screw or roller type) on the tubing pinches the tubing to regulate flow. Since the gum-rubber port is self-sealing, additional medication can be added to the IV system at these ports of entry. Glass containers that have no air tubes require air-inlet filters designed as part of the administration set (Abbott). See Figs 1 to 6.

Administration Procedures

In the administration of IV fluids, the primary IV container provides for fluid replacement, electrolyte replenishment, drug therapy or nutrition; the fluid can be infused over a 4- to 8-hour period. In some cases an IV fluid is infused slowly for the purpose of keeping the vein open (KVO). This will allow additional drugs to be administered when required. The primary IV fluid also can serve as a vehicle for other drugs to be administered, thus becoming an intravenous admixture (IV drip) and results in continuous blood levels of added drugs once the steady state has been reached.

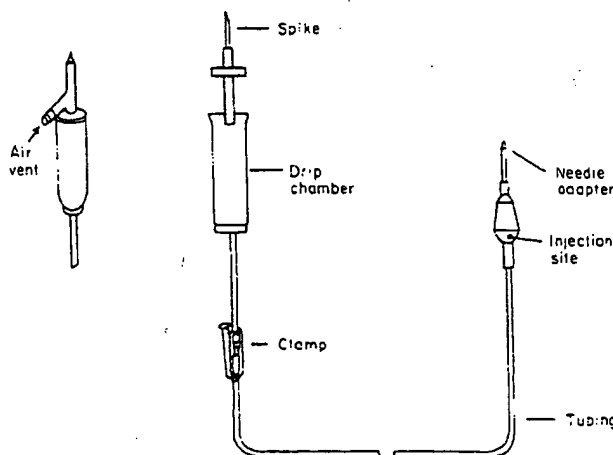


Fig 1. Parts of basic administration sets.

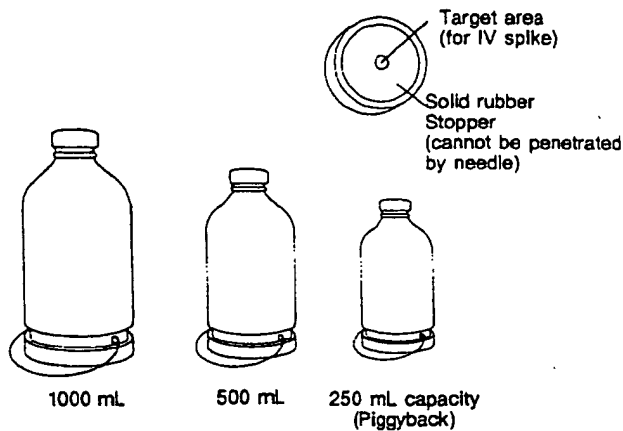


Fig 2. Abbott IV glass container. The air venting is provided through the air filter located in the spike of the administration set. See Fig 1.

The *Accumed* (McGaw) semirigid polyolefin plastic container is being phased out of production in favor of a new non-PVC, non-phthalate flexible container termed *Excell*.

Incinerated PVC products produce hydrogen chloride gas as a toxic pollutant. Diethyl hexyl phthalate (DEHP), a component of PVC containers may leach into the soil in landfills. A number of drugs adsorb on PVC containers, notably nitroglycerin. Some drugs (fat emulsions, blood, taxol) are known to leach DEHP.

The *Excell* container is claimed to eliminate or minimize these problems. The plastic film contains no plasticizers and exhibits no leachability. The solution-contact layer of the container is composed of a rubberized copolymer of ethylene and propylene; which is claimed to be clear, nontoxic and biologically inert. The container is available in 250-mL, 500-mL, and 1-L sizes. Smaller sizes are available in 25, 50 and 100 mL known as PAB containers.

In preparing an IV fluid for administration, the following procedure is used.

The spike adapter of the administration set is inserted into the stopper or seal of the IV container.

The IV fluid is hung on a stand at bedside and air is purged from the administration set by opening the clamp until fluid comes out of needle. The tubing is then clamped off.

The venipuncture is made by member of the IV team, floor nurse or physician.

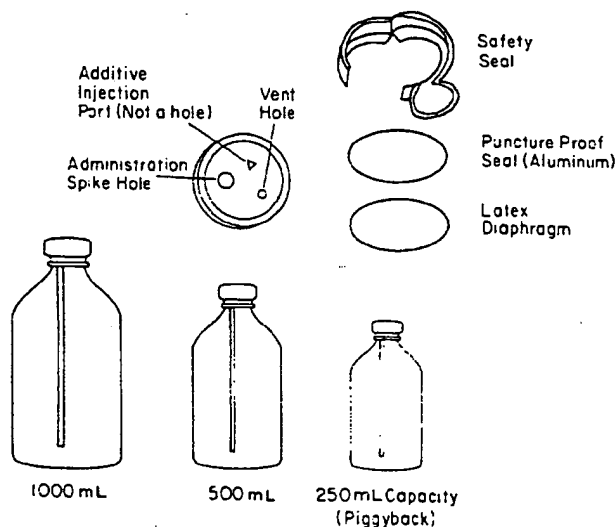


Fig 3. Baxter and McGaw glass containers. The plastic air tube allows the air to enter the bottle as the fluid is infused into the patient. The spike of the administration set is not vented. See Fig 1.

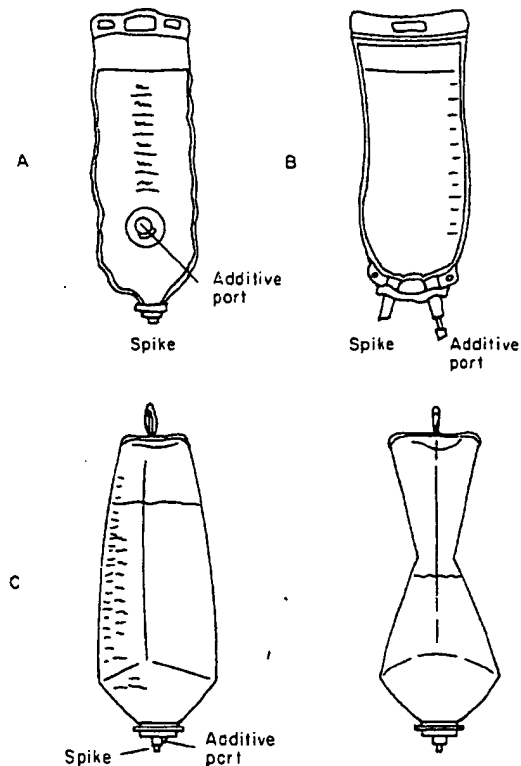


Fig 4. (A) Abbott (*Lifecare*) polyvinyl chloride flexible container; (B) Baxter (*Vialflex*) polyvinyl chloride flexible container. These containers take nonvented administration sets. See Fig 1.

The infusion rate is adjusted by slowly opening and closing the clamp until the desired drop rate, viewed in the drip chamber, is obtained. The usual running time is 4 to 8 hours (usually 125 mL are delivered in 1 hour). Drugs such as heparin, insulin, lidocaine or dopamine may be present in the IV drip. When potent drugs are present, the flow rates will vary depending on the clinical condition of the patient. Sets are calculated to deliver 10, 15, 20, 50 or 60 drops per mL, depending on the manufacturer.

Intermittent administration of an antibiotic and other drugs can be achieved by any of three methods: (1) direct intravenous injection (IV bolus or push), (2) addition of the drug to a predetermined volume of fluid in a volume-control device or (3) use of a second container (minibottle, minibag) with an already hanging IV fluid (piggybacking).

Direct Intravenous Injection—Small volumes (1 to 50 mL) of drugs are injected into the vein over a short period of time (1 to 5 minutes). The injection also can be made through a resealable gum-rubber injection site of an already hanging IV fluid. This method is suitable for a limited number of drugs but too hazardous for most drugs.

Volume-Control Method—Volume-control sets provide a means for intermittent infusion of drug solutions in precise quantities, at controlled rates of flow. These units consist of calibrated, plastic, fluid chambers placed in a direct line under an established primary IV container or more often attached to an independent fluid supply. In either case, the drug to be administered is first reconstituted if it is a sterile solid and injected into the gum-rubber injection port of the volume-control unit. It is then further diluted to 50 to 150 mL with the primary fluid or the separate fluid reservoir. Administration of the total drug-containing solution requires 30 to 60 minutes and produces a peak concentration in the blood followed by a valley if the dosage is discontinued. The following volume-control sets are available commercially: *Soluset*, Abbott; *Buretrol*, Baxter and *Metriset*, McGaw.

The procedure for setting up an intermittent IV infusion with a volume-control set is

Using aseptic technique, the spike of the volume-control set is inserted into the primary IV fluid or a separate fluid container. See Fig 6.

Air
clam:
Th
to 50
Th
Th
cont

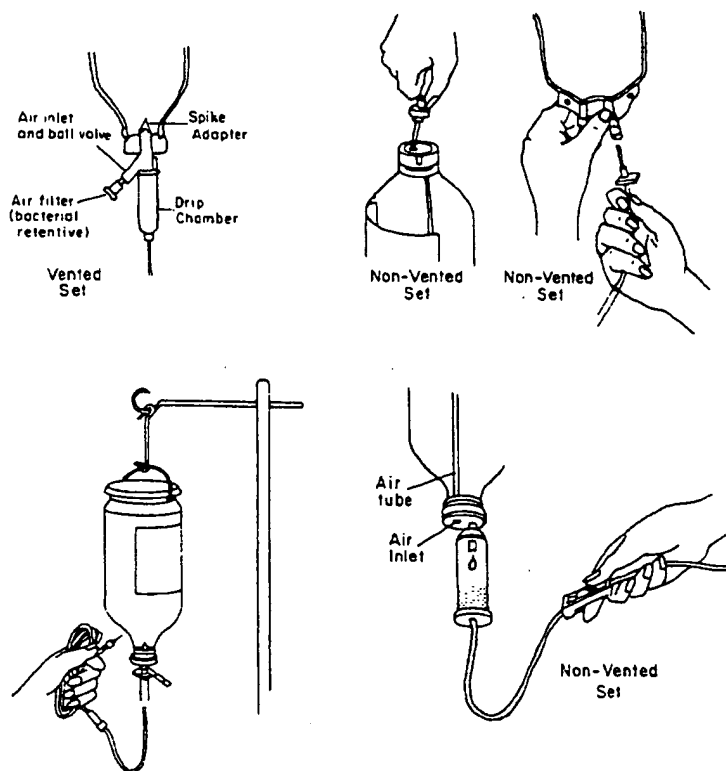


Fig 5. Setting up a primary IV fluid for administration.

Air is purged from tubing of the volume-control set by opening the clamps until fluid comes through.

The clamp is opened above the calibrated chamber and it is filled with 25 to 50 mL fluid from the primary IV container or separate fluid container.

The clamp is closed above the chamber.

The medication is injected through the gum-rubber port of the volume-control unit.

The clamp above the chamber is opened to complete the dilution to the desired volume (50 to 150 mL), then closed.

Flow commences when the clamp below the volume-control unit is opened.

Piggyback Method—The piggyback method (Fig 7) refers to the intermittent IV drip of a second solution, the reconstituted drug, through the venipuncture site of an established primary IV system. With this setup the drug can be thought of as entering the vein on "top" of the primary IV fluid, hence the designation "piggyback." The piggyback technique not only eliminates the need for another venipuncture, but also achieves drug dilution and peak blood levels within a relatively short time span, usually 30 to 60 minutes. Drug dilution helps to reduce irritation, and early high serum levels are an important consideration in serious infection requiring aggressive drug therapy. These advantages have popularized the piggyback method of IV therapy, especially for the intermittent administration of antibiotics. In using the piggyback technique, the secondary unit is purged of air and its needle inserted into a Y-injection site of the primary set or into the injection site at the end of the primary set. The piggyback infusion is then started. Once it is completed, the primary fluid infusion will be restarted. See Fig 7.

Primary IV administration sets are available that have a built-in check valve for use in piggyback administration. When the piggyback is connected to one of these sets and started, the check valve automatically closes off the primary infusion. When the piggyback runs out, the check valve automatically opens, thereby restarting the primary infusion. The check valve works because of pressure differences. To achieve this difference, the primary container is hung lower than the secondary bottle by means of an extension hanger. See Fig 8.

Manufacturers have introduced minibottles prefilled with various antibiotic products; each container is provided with a plastic hanger for direct suspension from an IV pole as the piggyback solution is administered through the resealable gum-rubber injection site or Y-type facility of an existing IV

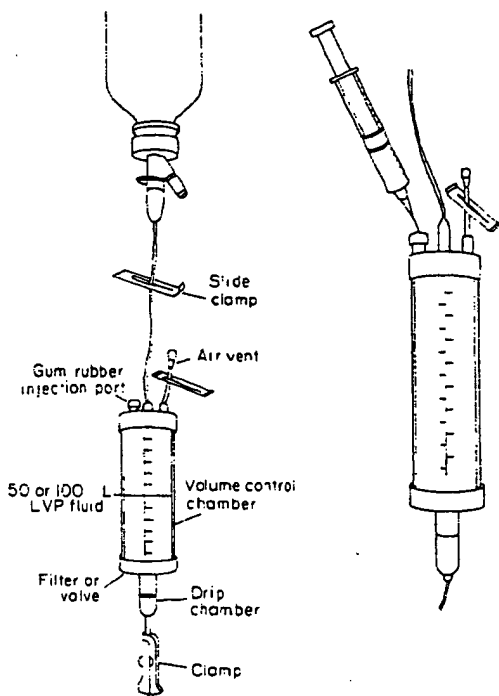


Fig 6. Volume control unit for intermittent administration.

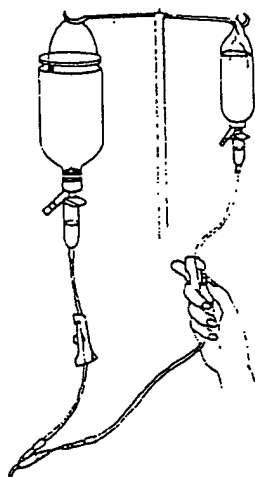


Fig 7. Piggyback administration setup.

system. Reconstitution of piggyback units requires only the addition of a small volume of compatible diluent. Since reconstitution and administration proceed from the same bottle, no drug transfer is involved, so transfer syringes and additional IV containers are not necessary. Prefilled drug containers offer significant advantages to hospitals. Time-saving, less potential for error and contamination and convenience are outstanding qualities of this type of packaging. The need exists in hospitals for these types of innovative packaging to help alleviate the critical nursing shortage and reduce the error potential. It is a significant event that drug manufacturers and intravenous fluid manufacturers have combined efforts to achieve optimal packaging for hospital use.

Partial-fill containers available for piggybacking are 250-mL capacity infusion bottles or bags underfilled with 50 or 100 mL D₅/W or normal saline. The drug to be administered first is reconstituted in its original parenteral vial and then added by needle and syringe to the partial-fill container. The needle of the piggyback delivery system is inserted into the Y-site or gum-rubber injection port of a hanging primary infusion set. Flow of the primary intravenous fluid is stopped while the drug solution in the partial-fill container is administered (30 to 60 minutes). After the drug solution has been

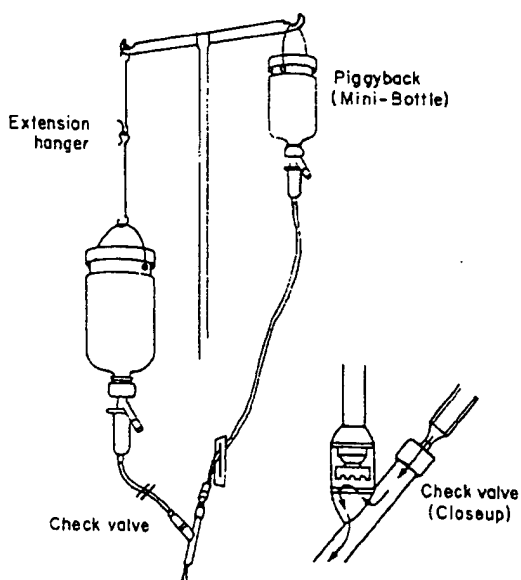


Fig 8. Piggyback administration setup with check valve in primary set.

infused totally, the primary fluid flow is re-established. When the next dose of drug is required, the piggyback procedure is repeated, replacing the prefilled partial-fill container.

Mechanical-Electronic Infusion Devices—Gravity IV administration systems are affected by many variables which tend to alter the accuracy of the system. These include variations in the size of the drip-chamber orifice, the viscosity of the solution being administered, plastic cold flow, clamp slippage, final filters, variations in the patient's blood pressure and body movements, clot formation, pressure changes in IV containers rate of flow, temperature of the IV fluid, changes in the needle and other factors such as kinked tubing, extravasation and changes in the height of the IV container. Flow in traditional gravity IV systems is controlled by manual clamps (either screw or roller clamps) which can provide considerable discrepancies in volume delivery. These factors have promoted the development and use of mechanical-electronic infusion devices to control more accurately the administration of IV fluids. This group of devices includes infusion controllers and infusion pumps.

Infusion controllers count drops electronically or extrude volumes of fluid mechanically and electronically. Having no moving components, controllers are less complex than pumps, being usually less expensive and having fewer maintenance problems. Infusion controllers are gravity-type systems, but the control is regulated automatically rather than manually. In addition to increasing the accuracy of delivery, electronic equipment may be able to detect infiltration of air, empty containers and excess or deficient flow.

Infusion pumps do not depend on gravity to provide the pressure required to infuse the drug. Pressure is provided by an electric pump that propels a syringe, a peristaltic or roller device or a cassette. Most pumps are volumetric in that the delivery is measured in milliliters rather than drops.

The quality of patient care has improved with the use of infusion devices. Flow rates can be maintained, therefore parenteral and enteral nutrition can be conducted safely. In addition, accurate drug therapy can be accomplished with adults and children and "runaways" of IV fluid administration can be eliminated.

Patient-Controlled Analgesia (PCA)—Usually and traditionally the acute or chronic pain experienced by patients in selected diseases are treated initially by oral narcotics and analgesics. However, many clinical situations preclude oral administration. Typically, the unsatisfied pain from disease has been treated by parenteral analgesics given by the IM or SC route.

This medication cycle from patient complaint to pain relief often can be lengthy. Frequently, the dose administered may be too large or too small, resulting in either sedation or poor pain relief. See Fig 10.

Parenteral drugs given IV offer rapid distribution in the body and fast onset of action; the drug undergoes no biotransformation or inactivation and, therefore, allows for more precise dose management.

Patient-Controlled Analgesia (PCA) is a system for delivery of IV or SC narcotics by direct patient intervention. This therapy uses a mechanical, electronic, infusion-control device which permits self-administration of analgesics in proportion to the degree of relief desired.

A number of these devices have been developed and are undergoing development at Bard, Abbott, Pharmacia, Deltec, Travenol and Becton Dickinson. The early devices allowed for patient-triggered IV doses, and later refinement in the microprocessors allowed for tailoring of infusions so that additional bolus doses could be given to a baseline infusion. Additional developments have led to ambulatory PCA devices that are small enough to be worn on a belt. An additional design being used is a balloon-powered disposable device (Travenol) that operates mechanically from an inflated balloon.

In its simplest terms, PCA allows a patient to initiate an IV infusion of a prescribed narcotic analgesic and maintain a

PCA

RESP
DEP

S

SERUM DRUG CONCENTRATION
AFig 9.
tratio

self-
cont
Th
equa
that
nurs
catic
perc
upon
in ce
Th
sion
P
drug
allow
K
intr
F
IV a
for
ture
adm
Cor

P

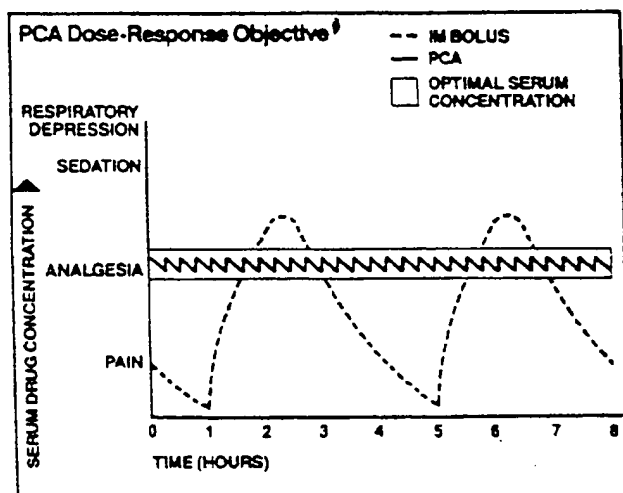


Fig 9. Characteristic pattern comparison of IM bolus serum concentration versus PCA.³

self-regulated small amount of incremental doses needed for controlling a variety of pain-associated medical problems.

The success and popularity of PCA is based upon the inadequacy of conventional IM and IV dosing, such as variables that effect absorption and distribution¹ such as conventional nursing practices, inherent procedural delays in securing medication and the ultimate administration to the patient.² The perception and sensation of pain in any one patient depends upon individual levels of endorphins and other biochemicals in cerebrospinal fluid.³

The last several years have seen the increasing use of infusion devices for epidural or intrathecal administration.

PCA eliminates the peak and valley effects of traditional drug therapy (Fig 9). Epidural or intrathecal therapy of PCA allows a longer duration of drug action.

Kwan⁴ reviewed the use of infusion devices for epidural or intrathecal administration.

Final-Filter Devices—Particulate matter in IV fluids and IV admixtures can originate from many sources. It can result from the packaging components of the IV fluid, from admixture incompatibilities, from manipulation in preparing the admixture and even from the administration set itself. Concern for particulate matter led to the design of final-filter

devices for attaching to the end of the tubing of the administration set. They afford a final filtration of the IV fluid before it passes through the needle into the vein. The device consists of a plastic chamber containing a membrane or stainless-steel filter having porosities varying from 5 to 0.22 μm . Air lock can be a problem with membrane filters. When wet, membranes with a porosity of 0.22 μm and 0.45 μm are impervious to air at normal pressures and air in the system causes blockage. In order to prevent this, the filter housing must be purged completely of air prior to use. Newer designs have air eliminators. Using final-filter devices increases medication cost but reduces the biological hazards associated with particulate matter.

Although considerable information is available concerning the clinical use of membrane filters in entrapping particulate matter and microorganisms, little information exists describing drug absorption by the filter. Literature on a limited number of drugs and filter materials indicates that drugs administered in low doses might present a problem with drug bonding to the filter.⁵ Solutions containing minute dosages of drugs, 5 mg or less, should not be filtered until sufficient data are available to confirm insignificant absorption. Drugs not recommended to be filtered include all parenteral suspensions, blood and blood products, amphotericin B, digitoxin, insulin, intravenous fat emulsions, mithramycin, nitroglycerin and vincristine.

IV Delivery Systems—

Frozen Premixes—Baxter provides delivery to hospitals of frozen drug products packaged in polyvinyl chloride containers. These are stored in a freezer in the hospital's pharmacy, thawed and used when needed. See Fig 11A.

Faspak/ADS-100 System—Eli Lilly supplies a non-PVC plastic piggyback container, named Faspak, which contains the dry, powdered form of certain drugs (Keflin, Kefzol, Mandol and ampicillin) which, upon reconstitution with the appropriate diluent, allows direct administration of the diluted drug. This avoids a transferring step that normally takes place when reconstituting a powdered drug. To help in the reconstitution step, a specialized dilution pump named the ADS-100 system is supplied. The package design eliminates the need for transferring between containers after reconstitution, and the Faspak acts as a final delivery container.

Abbott/ADD-Vantage System—Introduced in 1985, the Abbott ADD-Vantage system (Fig 11B) has two parts: a plastic IV bag (Abbott) which is filled with solution and a separate glass vial of powder or liquid drug sold by a pharmaceutical manufacturer. The vial is encased by a plastic cover that is removed prior to use. The user locks the vial holding the drug into a chamber at the top of the plastic bag and mixes the drug and solution by externally removing the stopper on the vial.

Nutrimix—A Dual-Compartment container is available from Abbott. This container allows for long-term packaging of amino acids and dextrose mixtures.

IVAC-Cris—The IVAC-Cris (Controlled-Release Infusion System) (Fig 11F) is a disposable adapter designed to infuse reconstituted injectable drugs directly from the manufacturer's single-dose vial. The Cris adapter avoids the need to transfer drug doses to piggyback secondary containers and also eliminates the need for a secondary IV set. The adapter has a primary spike that is inserted into the IV fluid container and a secondary spike that receives the drug vial. The vial spike has two fluid paths: one admits IV fluid from the primary container into the vial; the other drains drug solution into the drip chamber of the IV set. A two-position valve allows IV fluid to flow directly from the primary container to the patient or pass through the vial to deliver the drug. A 5 μm in-line filter eliminates particulates.

To operate the Cris adapter, the drug vial first is reconstituted with an appropriate diluent. With the valve dial in the vertical (primary) position, the spike shield is removed and the vial is attached immediately to the Cris spike. The valve dial is then turned toward the vial, directing the flow of primary fluid into the vial of drug solution. The incoming fluid

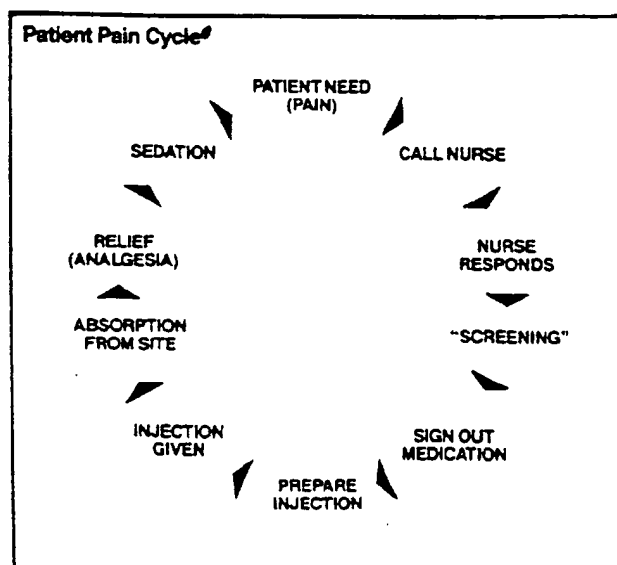


Fig 10. Patient pain cycle—sequence of events.³

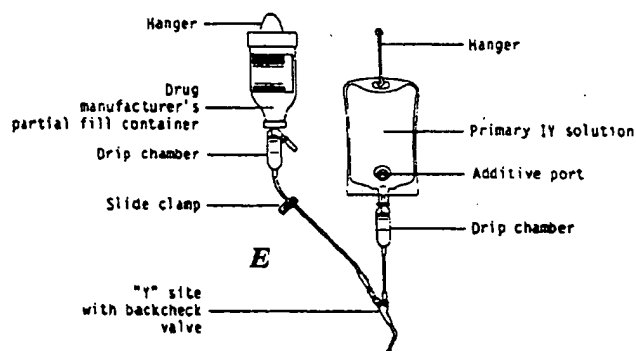
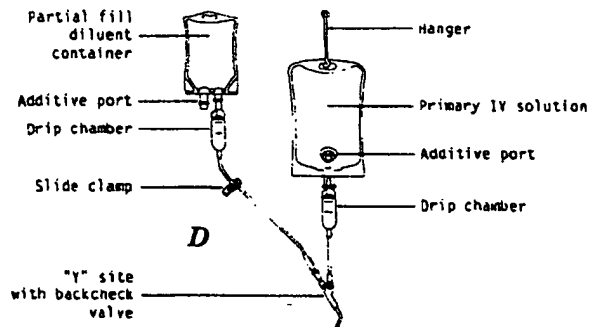
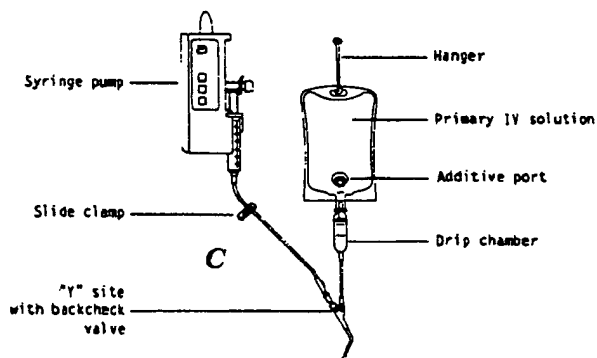
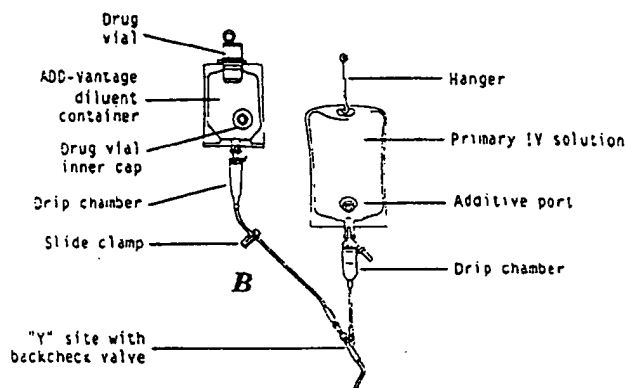
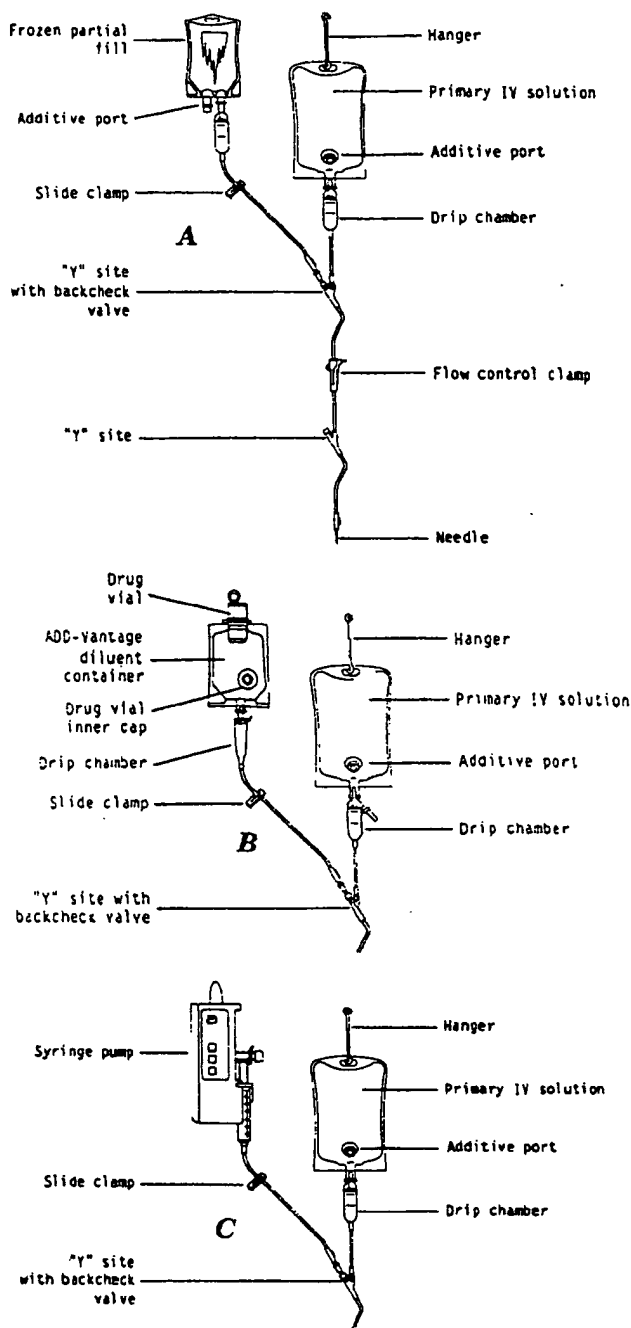


Fig 11. Various IV delivery systems. A: Frozen partial fill; B: Add-Vantage; C: syringe pump; D: partial fill diluent container; E: drug manufacturer's partial fill piggyback (DMP) (courtesy, Abbott). The flow control clamp, "Y" site, needle and associated tubing for B through E, are the same as in A. (Fig 11 is continued on the next page.)

dilutes and displaces the drug solution into the drip chamber, through the primary set and into the patient. After the dose has been delivered, the vial remains on the spike until the next dose is required. Flow rate can be adjusted using a roller clamp, electronic pump or controller.

Mini-Infuser Pumps for Intermittent IV Drug Delivery—A novel concept in intermittent drug delivery, introduced several years ago, was the Bard-Harvard Mini-Infuser System. This instrument was designed for the administration of antibiotics and other medications delivered intermittently in 40 minutes or less. This battery-generated, lightweight instrument uses standard disposable syringes and microbore disposable extension sets. Different models are available, depending on volume-to-be-delivered selection. This instrument provides accuracy, constant flow, convenience and safety for intermittent drug delivery. See Fig 11C.

Introduced and designed for intermittent IV drug delivery, Becton Dickinson's 360 Infusor allows drug delivery intermittently over 60 minutes or less in a volume dilution of up to 60 mL.

Internal Methods Used To Achieve Intravascular Access—

Implantable Ports (Infuse-A-Port, Infusaid; Port-A-Cath, Pharmacia)—Broviac and Hickman catheters have been used to achieve long-term venous access in a variety of diseases. Although these catheters are widely used, they are associated with some morbidity which includes fracture of catheters entrance site infection and catheters sepsis. Implantable catheters have been developed to overcome catheter complications and are designed to permit repeated access to the infusion site. The catheters consist of implantable-grade silicone tubing connected to a stainless-steel port with a self-

seal
eter
syst
into
the
T
ang

Fi
m

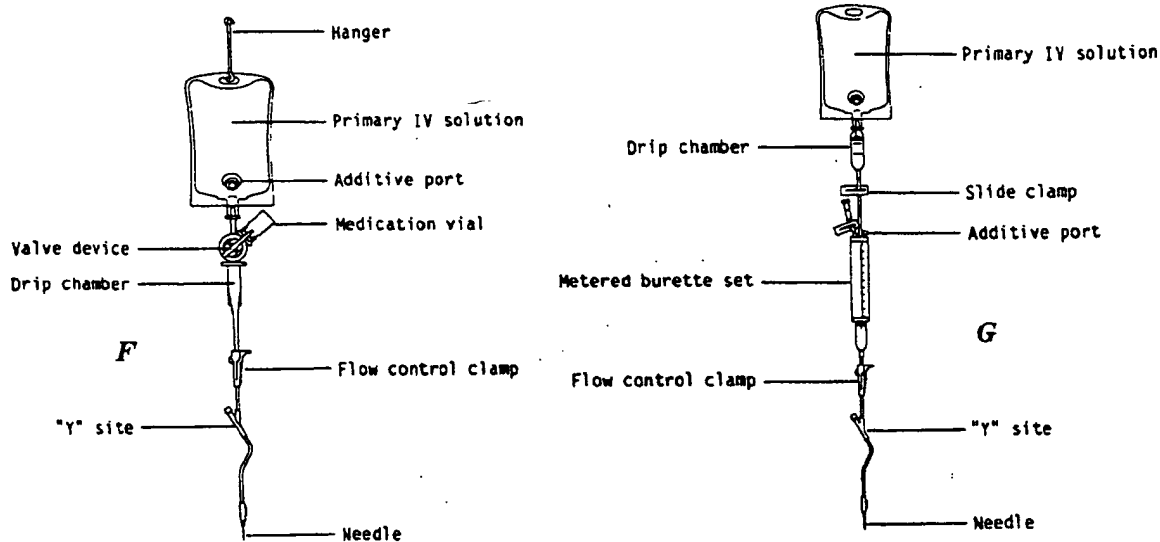


Fig 11 (continued). F: Cris infusion; G: burette set (courtesy, Abbott).

sealing septum that allows needle access. The delivery catheter can be placed in a vein, cavity, artery or the CNS. The system is accessed with a Huber-point needle through the skin into the self-sealing silicone plug positioned in the center of the portal.

The specialized Huber-point needle is designed with an angle bevel that reduces coring and permits easy entry.

These implantable ports can be used for the injection of IV fluids, total parenteral nutrition, chemotherapy, antibiotics and other drugs.

Some advantages of implantable devices include

- The need for a long-term access site to venous, arterial and spinal systems.
- An increased dependence on *nonhospital* treatment of chronic disease states.
- The direct infusion in a target organ or tumor.
- A decrease in infection rates which are seen with percutaneous catheters or repeated spinal taps.
- Allowing greater mobility for the patient (a return to normal function).

Implantable Pump (Infusaid)—The Infusaid Implantable Pump was approved for selected drug administration. This pump is the size of a hockey puck and weighs approximately 6½ oz. The construction is titanium, stainless steel and polypropylene. The injection part is constructed of silicone rubber and has a usable life of at least 2000 punctures. Under normal use this device lasts more than 8 years.

The internal power supply uses Freon in equilibrium between the gaseous and liquid states and is *recharged* with each refilling process, thus supplying a power supply for as long as the pump is needed. As the pump is refilled, it compresses the gas back into the liquid state, allowing a fresh supply of energy for the next cycle. The capacity of this pump is 50 mL, which can be administered over a 14-day period. The pump accuracy is stated as over 3%. The cost of one model is approximately \$4000.00, not including the surgical implant procedure. The 14-day cycle cannot be altered to any degree.

Model 400 Implantable Drug Delivery System (Infusaid) is designed for long-term therapy in the ambulatory patient. The Model 400 with a 47-mL usable drug volume delivers a precise, continuous flow to a selected organ or site via a soft, nontraumatic, nonthrombogenic silicone rubber catheter. The Model 400 also features an auxiliary *Sideport* septum, completely bypassing the pumping mechanism, for delivery of direct bolus injections to the target site. This allows the clinician easily to supplement the continuous infusion with additional drugs, objectively assess the disease state or monitor catheter location and drug perfusion with the use of radio-labeled microspheres.

Intravenous Admixtures

When one or more sterile products are added to an IV fluid for administration, the resulting combination is known as an



Fig 12. Placing an additive into an IV fluid with filtration through a membrane filter (courtesy, Millipore).

IV admixture. To maintain the characteristics of sterile products, namely sterility, freedom from particulate matter and pyrogens, it is imperative that they be manipulated in a suitable environment using aseptic techniques.

Environment—Proper conditions for aseptic handling can be provided by laminar-flow hoods (see Chapters 84, 87). Within a laminar-flow hood, air filtered through a HEPA (high efficiency particulate air) filter moves in a parallel flow configuration at a velocity of 90 fpm. HEPA filters remove 99.97% of all particles larger than 0.3 μm . Since microbial contaminants present in air usually are found on other particulates, removal of the latter results in a flow of air free of both microbial contaminants and particulate matter. The movement of the filtered air in a laminar-flow configuration at a velocity of 90 fpm can maintain the area free of contamination. The flow of air may be in either a horizontal or vertical pattern. In the former case the HEPA filter is located at the back of the hood and the air flows to the front. In vertical flow the air passes through the HEPA filter located in the top of the cabinet and is exhausted through a grated area around the working surface of the hood. Regardless of the type of laminar air flow, the hood must be operated and maintained properly in order to achieve a satisfactory environment for the preparation of parenteral admixtures.

The hood is situated best in a clean area in which there is little traffic flow past the front of the hood. The inside of the hood is wiped down thoroughly with a suitable disinfectant and allowed to run for at least 30 minutes before starting manipulations. It is important to remember that the laminar-flow hood is not a means of sterilization. It only maintains an area free of microbial contaminants and particulate matter when it has been prepared, maintained and utilized properly by operators having proper aseptic techniques.

Before working in a laminar-flow hood the operator washes his hands thoroughly and scrubs them with a suitable disinfectant. Some laboratories may require gowning and using sterile gloves. Sterile gloves can be an asset but there is always the problem that they can give the operator a false sense of security. Gloved hands can become contaminated as easily as ungloved hands. Additives and IV fluids to be used in the preparation of the admixture, along with suitable syringes, are lined up in the hood in the order they are to be used. The containers must be clean and dust-free. They are inspected for clarity and freedom from cracks. Operators are encouraged to use a lighting device for inspecting IV fluids for particulate matter and cracks. The lighting device should permit the container to be viewed against both a light and a dark background during inspection. If the IV fluid is packaged in plastic containers, pressure is applied to assure that they are sealed properly and do not leak. Some laboratories disinfect the containers prior to placing them in the hood.

In working within the hood the operator works in the center of the hood, with the space between the point of operation and the filter unobstructed. If the flow of air is blocked, the validity of the laminar flow is destroyed. Articles are arranged within the hood in a manner to prevent clean air from washing over dirty objects and contaminating other objects that must remain sterile. The working area must be at least 6 inches from the front edge of the hood. As the operator stands in front of the hood, his body acts as a barrier to the laminar air flow causing it to pass around him and create backflow patterns which can carry room air into the front of the hood.

Laminar-flow hoods must be maintained and evaluated periodically to insure that they are functioning properly. The velocity of air flow can be determined routinely using a velometer. A decrease in the air flow usually indicates a clogged HEPA filter. Some laminar-flow hoods are equipped with pressure gauges indicating pressure in the plenum behind the filter; in these hoods pressure increase also can indicate a clogged filter. Settling plates can be exposed within the hood for given periods of time to determine the presence of microbial contaminants.

The best way to determine the proper functioning of a HEPA filter is to use the dioctyl phthalate (DOP) test using the vapor at room temperature. DOP vapor (particles of $\sim 0.3 \mu\text{m}$) is allowed to be taken up by the hood through its intake filter. If the HEPA filter is intact and properly installed, no DOP can be detected in the filtered air stream using a smoke photometer. Certification services are available through commercial laboratories; the HEPA filters within laminar-flow hoods should be evaluated every 6 months.

Additives—The additives are injections packaged in ampuls or vials, or sterile solids; the latter are reconstituted with a suitable diluent before addition to the IV fluid. A fresh, sterile, disposable syringe is used for each additive. Before removing a measured volume from an ampul, the container is wiped with a disinfectant solution. If the ampul is scored, the top can be snapped off; if not scored, an ampul file must be used. A sterile syringe is removed from its protective wrapping. The syringe needle with its cover is separated from the syringe aseptically and may be replaced with a sterile aspirating needle. Aspirating needles usually are made from clear plastic and contain a stainless-steel or nylon filter having a porosity of 5 μm . The filter will remove glass particles and other particulates from the injection as it is drawn up from the ampul into the syringe. The aspirating needle is replaced with the regular needle. The exact volume is calibrated and the injection is ready to be added to the IV fluid (see Fig 12). In the case of additives packaged in multiple-dose vials, the protective cover is removed and the exposed target area of the rubber closure disinfected. A volume of air, equal to the volume of solution to be removed, is drawn up into the syringe and injected into the air space above the injection within the vial. This facilitates withdrawal of the injection. The solution is drawn into the syringe, the exact dose is measured and the injection is ready to be added to the IV fluid.

Certain injections are light-sensitive and protected against photolysis by the container packaging. The manufacturer may use amber glass, individual container wrapping or an amber plastic cover. Many hospital pharmacists use aluminum foil as a protective wrap for light-sensitive drugs during their administration.

In the case of drug substances having poor stability in aqueous solution, the drug is packaged as a sterile solid, either dry-filled or lyophilized. The diluent recommended on the labeling is used to reconstitute the powder; the proper quantity of solution then is removed for addition to the IV fluid. When large volumes of diluent are required for reconstitution, as for Keflin, a sterile needle is placed through the closure to vent the container and facilitate addition of the diluent. In order to increase the efficiency of IV admixture programs, a limited number of hospital pharmacists have found it convenient to freeze reconstituted drugs, particularly antibiotics. The stability of reconstituted drugs is somewhat limited. In some cases stability is limited to only a few hours; in many cases, however, reconstituted solutions can be frozen and thawed at the time of use. In the frozen form the stability of the antibiotic solution can be increased. In a number of instances the stability in the frozen form is known and supplied by the manufacturer. Reports have been published on the frozen stability of certain drugs. However, it is unwise to freeze drug solutions without adequate stability studies for guidance. In those cases where published information is available, close adherence must be observed as to freezing temperature, storage conditions and packaging.

There is an increasing awareness of the potential hazard to pharmacists handling antineoplastic drugs.⁶ Although the evidence is not conclusive, it appears that measures should be taken to minimize unnecessary exposure.⁷ These precautions include the use of vertical laminar-flow hoods for the preparation and reconstitution of these agents, the wearing of gloves and masks by the personnel, special labeling of the containers to insure their proper handling and disposal and periodic blood studies of personnel involved in preparing admixtures of antineoplastic agents.

T
dep
by t
in T.

1.
with
2.
3.
The
4.
5.
is nc

1.
late:
2.
drav
3.
is th
inje
4.
cov
drav
vac
pric
5
6
adr

1
plu
2
ove
3
4
ava
5
ins

rut
the
mi

ag
si
tu
lt
ti
aq
rr
q
a

ti
n
s
P
tl
t
r
li
t

The procedure for placing an additive in an IV fluid will vary depending on the type of IV fluid packaging system being used by the hospital. The packaging systems have been described in Table 2.

Abbott Glass Containers (Fig 2)

1. Remove the aluminum tear seal exposing the solid-rubber closure with a target circle in the center.
2. Wipe the closure with suitable disinfectant.
3. Insert the needle of the additive syringe through the target area. The vacuum within the bottle draws in the solution.
4. Gently shake the bottle after each addition.
5. When completed, cover the closure with a plastic protective cap if it is not to be used immediately.

Baxter and McGaw Rigid Glass Containers (Fig 3)

1. Remove the aluminum tear seal and the aluminum disc covering the latex diaphragm.
2. Upon exposing the latex diaphragm, note that the latex cover is drawn in over the openings in the rubber closure.
3. The larger of the two holes receives the administration set, the other is the air vent. The triangle-shaped indentation can serve as the site for injecting the additives as well as the opening for the administration set.
4. Wipe the diaphragm with a suitable disinfectant and pierce the latex cover to place additive into bottle. The vacuum within the bottle will draw additive from the syringe. Do not remove the diaphragm or the vacuum will dissipate. It will be removed at the time of administration prior to the insertion of the administration set.
5. Gently shake the bottle after each additive.
6. When completed, cover the bottle with a plastic additive cap if the administration set is not to be inserted immediately.

Baxter and Abbott Plastic Container (Fig 4)

1. Remove the additive port protective sleeve and rub the gum-rubber plug with a suitable disinfectant.
2. Additives are placed in container by piercing the gum-rubber cover over the additive port.
3. After each addition, milk the container to insure adequate mixing.
4. Containers do not contain a vacuum, but vacuum chambers are available for use in conjunction with the flexible plastic container.
5. Protective additive caps are available if the administration set is not inserted immediately.

McGaw Semirigid Plastic Container (Fig 4)

1. Remove the additive port protective covering and rub the gum-rubber plug with a suitable disinfectant.
2. Additives are placed in containers by piercing the gum-rubber over the additive port.
3. After each addition, shake the container gently to insure adequate mixing.
4. Containers do not contain a vacuum.

Pharmacy Bulk Package—The manufactured bulk package is a sterile container for parenteral use that contains many single doses. These containers are intended for use in admixture programs where large numbers of doses are prepared. It is designed so that the rubber closure is penetrated only one time. It is used in laminar-flow hoods. Pharmacy bulk packages are exempt from the USP requirement that requires multiple-dose containers to have a volume not greater than 30 mL. They also have an exemption in that they are not required to have a bacteriostatic agent. Pharmacy bulk packages have special labeling and storage requirements.

Parenteral Incompatibility—When one or more additives are combined with an IV fluid, their presence together may modify the inherent characteristics of the drug substances present, resulting in a parenteral incompatibility. Parenteral incompatibilities have been divided arbitrarily into three groups: physical, chemical and therapeutic. The latter is the most difficult to observe because the combination results in undesirable antagonistic or synergistic pharmacologic activity. For example, the report that penicillin or cortisone antagonizes the effect of heparin and produces a mis-

leading picture of the anticoagulant effect of heparin represents a therapeutic incompatibility. Physical incompatibilities are observed most easily and can be detected by changes in the appearance of the admixture, such as a change in color, formation of a precipitate or evolution of a gas. Physical incompatibilities frequently can be predicted by knowing the chemical characteristics of the drugs involved. For example, the sodium salts of weak acids, such as phenytoin sodium or phenobarbital sodium, precipitate as free acids when added to intravenous fluids having an acidic pH. Calcium salts precipitate when added to an alkaline medium. Injections that require a special diluent for solubilization, such as diazepam, precipitate when added to aqueous solutions because of their low water solubility.

Decomposition of drug substances resulting from combination of parenteral dosage forms is called a chemical incompatibility, an arbitrary classification since physical incompatibilities also result from chemical changes. Most chemical incompatibilities result from hydrolysis, oxidation, reduction or complexation and can be detected only with a suitable analytic method.

An important factor in causing a parenteral incompatibility is a change in the acid-base environment.⁸ The solubility and stability of a drug may vary as the pH of the solution changes. A change in the pH of the solution may be an indication in predicting an incompatibility, especially one involving drug stability, since this is not necessarily apparent physically. The effect of pH on stability is illustrated in the case of penicillin. The antibiotic remains active for 24 hours at pH 6.5, but at pH 3.5 it is destroyed in a short time. Potassium penicillin G contains a citrate buffer and is buffered at pH 6.0 to 6.5 when reconstituted with Sterile Water for Injection, Dextrose Injection or Sodium Chloride Injection. When this reconstituted solution is added to an intravenous fluid such as Dextrose Injection or Sodium Chloride Injection, the normal acid pH of the solution is buffered at pH 6.0 to 6.5, thus assuring the activity of the antibiotic.

While it may be impossible to predict and prevent all parenteral incompatibilities, their occurrence can be minimized. The IV admixture pharmacist should be cognizant of the increasing body of literature concerning parenteral incompatibilities. This includes compatibility guides published by large-volume parenteral manufacturers,⁹⁻¹¹ compatibility studies on individual parenteral products by the manufacturer and published with the product as part of the labeling, the study of the National Coordinating Committee on Large-Volume Parenterals,¹² reference books^{13,14} and literature reports of studies with specific parenteral drugs.¹⁵ The pharmacist should encourage the use of as few additives as possible in IV fluids since the number of potential problems increases as the number of additives increases. Physicians should be made aware of possible incompatibilities and the pharmacist can suggest alternate approaches to avoid the difficulties. In some instances, incompatibilities can be avoided by selecting another route of administration for one or more of the drugs involved.

Quality Control—Each hospital should have written procedures covering the handling and storage, use in preparing admixtures, labeling and transportation of IV fluids to the floors. In-use clarity and sterility tests should be devised to assure that IV admixtures retain the characteristics of sterility and freedom from particulate matter. Training and monitoring personnel involved in preparation of IV admixtures should be done on a regular basis.¹⁶ The efforts of the hospital pharmacy should be no less than those of the industry in following Current Good Manufacturing Practice to assure the safety and efficacy of these compounded medications.

Total Parenteral Nutrition

Intravenous administration of calories, nitrogen and other nutrients in sufficient quantities to achieve tissue synthesis and anabolism is called total parenteral nutrition (TPN).¹⁷

Originally, the term hyperalimentation was used to describe the procedure, but it is being replaced by TPN, the latter being more descriptive for the technique.

The normal caloric requirement for an adult is approximately 2500 per day. If these were to be provided totally by D5/W, approximately 15 L would be required. Each liter contains 50 g dextrose, equivalent to 170 calories. However, it is only possible to administer 3 or 4 L per day without causing fluid overload. To reduce this fluid volume the concentration of dextrose would have to be increased. By increasing the dextrose to 25%, it is possible to administer five times the calories in one-fifth the volume. D25/W is hypertonic and cannot be administered in large amounts into a peripheral vein without sclerosing the vein.

Dudrick developed the technique for administering fluids for TPN by way of the subclavian vein into the superior vena cava where the solution is diluted rapidly by the large volume of blood available, thus minimizing the hypertonicity of the solution. For administration of the TPN fluids, a catheter is inserted and retained in place in the subclavian vein. TPN is indicated in patients who are unable to ingest food due to carcinoma or extensive burns; patients who refuse to eat, as in the case of depressed geriatrics or young patients suffering from anorexia nervosa and surgical patients who should not be fed orally.

The preferred source for calories in TPN fluids is the carbohydrate dextrose. In IV fluid kits commercially available for the preparation of TPN solutions, D50/W is provided. On

dilution with amino acid injection, the resulting dextrose concentration is approximately 25%. It is this concentration that is administered.

The source of nitrogen in TPN fluids is crystalline amino acids (*Aminosyn*, Abbott; *FreAmine III*, McGaw; *Travasol*, Travenol). The crystalline amino acid injections contain all the essential and nonessential amino acids in the L-form. For optimum utilization of amino acids and for promoting tissue regeneration, the nitrogen-to-calorie ratio should be 1:150. Calories are needed to provide energy for the metabolism of nitrogen.

Electrolyte requirements vary with the individual patient. The electrolytes present in Amino Acid Injection are given on the label and must be taken into consideration in determining the quantities to be added. Usual electrolyte concentrations are required to fall within the following ranges: sodium, 100–120 mEq; potassium, 80–120 mEq; magnesium, 8–16 mEq; calcium, 5–10 mEq; chloride, 100–120 mEq and phosphate, 40–60 mEq. It is better to keep a 1:1 ratio between sodium and chloride ions. In adding potassium, the acetate salt is preferred to the chloride. If the combination of calcium and phosphate ions exceeds 20 mEq, precipitation occurs.

In addition to the electrolytes, the daily requirement for both water-soluble and fat-soluble vitamins may be added, usually in the form of a multivitamin infusion concentrate. Iron, should be administered separately from the TPN fluids. Trace elements such as zinc, copper, manganese and iodide

Table 3—Typical IV Orders (Parenteral Prescriptions)

Prescription	Comment	Prescription	Comment
1. R NS 1000 mL 125 mL/hr	Sodium Chloride Injection (Normal Saline Solution) 1000 mL, is to be administered at a flow rate of 125 mL per hr. It will require approximately 8 hr.	7. R 1000 cc TPN (FreAmine) + 40 mEq NaHCO ₃ + 30 mEq KCl + Vits + 5U Reg Insulin to run 80 cc/hr	One L of the basic TPN solution, FreAmine II, is to be provided with the addition of 40 mEq NaHCO ₃ , 30 mEq potassium chloride, the contents of one container vitamin B complex with vitamin C plus 5 units of regular zinc insulin. It is to be administered at the flow rate of 80 mL per hr (approximately 12 hr).
2. R 1000 D5W + NS + vits 12 hr	Dextrose Injection 5%, 1000 mL, containing 0.9% sodium chloride and container of vitamin B complex with vitamin C is to be administered over a 12-hr period.	8. R 1000 TPN + 40 mEq NaCl + 10 KCl + 10 Insulin + 10 cal gluconate	One L of the hospital's basic TPN solution is to be provided with the addition of 40 mEq sodium chloride, 10 mEq potassium chloride, 10 units regular zinc insulin and 10 mL Calcium Gluconate Injection.
3. R 500 D5W + ½NS KVO	Dextrose Injection 5%, 500 mL, containing 0.45% sodium chloride is to be administered at a flow rate to keep the vein open (KVO). The flow rate will be approximately 10 mL per 1 hr.	9. R Keflin 2 g + 100 mL D5W q 6 hr	Cephalothin, 2 g, is reconstituted with Sterile Water for Injection and added to a minibottle containing 100 mL Dextrose Injection 5%. This dose is given every 6 hr using a piggyback technique with a flow rate requiring 30 to 60 min for delivery.
4. R 1000 cc D5W + ½NS Add 1 amp vits to each + 100 mg thiamine Each to run 6 hr	Dextrose Injection 5%, 1000 mL, containing 0.45% sodium chloride, the contents of one ampul vitamin B complex with vitamin C and sufficient volume of Thiamine Hydrochloride Injection to give 100 mg thiamine, is to be administered over a 6-hr period (approximately 170 mL per hr). Additional orders of the same can be anticipated.	10. R Gentamicin 80 mg IVPB q 8 hr	Gentamicin, 80 mg, is added to a minibottle containing 100 mL Dextrose Injection 5%. This dose is given every 8 hr using the piggyback technique (IVPB) with a flow rate requiring at least 80 min (not less than 1 mg per min).
5. R 1000 cc D5W + ½NS + 20 mEq KCl	Dextrose Injection 5%, 1000 mL, is to be provided containing 0.45% sodium chloride and 20 mEq potassium chloride.		
6. R 1000 Hyperal + 10 NaCl - 10 KCl + 5 MgSO ₄ - 10 insulin	One L of the hospital's basic TPN solution is to be provided with the addition of 10 mEq sodium chloride, 10 mEq potassium chloride, 5 mEq magnesium sulfate and 10 units regular zinc insulin.		

Tre

Hum
Protu
Hum
Rofe
Intrc
Acti
Rec
Eng
Orth
Epo

are a c
requir

The F

The
scripti
chart.
comp
the in
tions,
order
pharm
fatic
admix
period
write
conta
conse
presc
ume
comp
are gi
prepa
expir
The
clude
work
will
prepa
from
name
tion
and
or dr
hang
taine
Th
viser
niqu
com
the f
orde
the
colo

Tabl 4—Product Stability

Trade name	Physical form	Shelf-life
Humulin	Liquid solution	2 yr at 2–8°C
Protopin	Lyophilized powder	2 yr at 2–8°C
Humatrope		
Roferon-A	Lyophilized powder	3 yr at 2–8°C
Intron A	Lyophilized powder	2 yr at 2–8°C
Activase	Lyophilized powder	2 yr at 2–30°C
Recombivax-HB	Liquid solution	
Engerix-B	Liquid solution	
Orthoclone	Liquid solution	1 yr at 2–8°C
Epogen	Liquid solution	

are a concern only in long-term cases and can be added when required.

Th Parenteral Prescription

The physician writes an admixture order or parenteral prescription on a physician's order form located on the patient's chart. A copy of the order is sent to the pharmacy for compounding. It includes the patient's name, room number, the intravenous fluid wanted, additives and their concentrations, rate of flow, starting time and length of therapy. The order is taken by the technician, nurse or pharmacist to the pharmacy. Orders may be telephoned to the pharmacy; verification with the original order is made on delivery of the admixture. IV orders usually are written for a 24-hour therapy period; the patient's chart is reviewed and new orders are written on a daily basis. The order may be for multiple containers, in which case the containers are numbered consecutively. Unlike the extemporaneously compounded prescription, additives are added without regard to final volume of IV fluid. The prescription is checked for proper dose, compatibility, drug allergies and stability. Additives usually are given an expiration period of 24 hours from the time of preparation. Drugs such as ampicillin may require shorter expiration periods.

The clerical work for the admixture is prepared. This includes typing of the label and the preparation of the profile worksheet. The profile sheet is filed so that the pharmacist will be alerted when subsequent containers are due for preparation. Charging the patient's account can be done from the profile worksheet. The label includes the patient's name, room number, bottle number, preparation date, expiration time and date, intravenous fluid and quantity, additives and quantities, total time for infusion, the milliliters per hour or drops per minute and space for the name of the nurse who hangs the container. The label will be affixed to the container upside down in order that it can be read when hung.

The admixture is prepared by the pharmacist or a supervised technician. In handling sterile products, aseptic techniques as discussed previously must be observed. When completed, a plastic additive cap is affixed before delivery to the floor. The label is applied and checked with the original order. The empty additive containers are checked to confirm the additives present. The admixture is inspected for any color change or particulate matter.

Table 5—Stability after Reconstitution (Lyophilized Products)

Trade name	Shelf-life
Roferon	1 mo at 2–8°C
Intron A	1 mo at 2–8°C
Humatrope	14 days at 2–8°C
Protopin	7 days at 2–8°C
Activase	8 hr at 2–30°C

Table 6—R combinant Protein Drug

Trade name	Vial strength
Humulin	1000 units
Protopin	5 mg
Humatrope	5 mg
Roferon-A	3 and 18 million units solution
	3 and 18 million units lyo
Intron A	3, 5, 10, 25 and 50 million units
Activase	20, 50 mg
Recombivax HB	5, 10 µg
Engerix-B	20 µg
Orthoclone OKT3	5 µg
Epogen	2, 4 and 10 thousand units

The completed admixture is delivered to the floor. If it is not to be infused immediately (within 1 hour), it is stored under refrigeration; if refrigerated, it must be used within 24 hours. The nurse checks for accuracy of patient's name, drug and concentration, IV fluid, expiration date, time started and clarity. The infusion of admixtures may run ahead or behind schedule, necessitating that the pharmacist modify the preparation of continued orders. Examples of IV orders are shown in Table 3.

Parenterals Derived by Biotechnology

In 1993, 14 biotechnology drugs had been approved for clinical use; 21 were in Phase III clinical studies awaiting approval and over 130 were in various phases of development. The Center for Biologics Evaluation and Review (CBER) had over 3200 INDs under review.

As a result of the stability sensitivities of proteins, the 14 biotechnology pharmaceuticals currently available are all manufactured as parenterals. Many are available as lyophilized parenterals (Table 4). Most have limited shelf-life after reconstitution (Table 5). All are supplied in low dosage, which attest to their potency (Table 6).

For a complete treatment of biotechnology and drugs, see Chapter 49.

References

1. Bennett RL, Griffen WO: *Contemp Surg* 29: 75, 1983.
2. Graves DA et al: *Ann Intern Med* 99: 360, 1983.
3. Blivins BA, Baumann TJ: *Patient Controlled Analgesia (PCA): A Clinical Evaluation of Safety and Efficacy in Hospitalized Trauma / Surgery Patients*, Depts of Surg and Pharm, Henry Ford Hospital, Detroit, Dec, 1984.
4. Kwan JW: *Am J Hosp Pharm* 47: 18, 1990.
5. Turco SJ: *Am J IV Ther. Clin Nutr* 9: 6, 1982.
6. Zimmerman PF, et al: *Am J Hosp Pharm* 38: 1693, 1981.
7. Gallelli JF: *Ibid* 39: 1877, 1982.
8. Newton DW: *Ibid* 35: 1213, 1978.
9. King JC: *Guide to Parenteral Admixtures*. Cutter Laboratories, Berkeley CA, 1987.
10. Shoup LK, Goodwin NH: *Implementation Guide—Centralized Admixture Program*. Travenol Laboratories, Morton Grove IL, 1977.
11. *Good IV Procedures Manual*. Abbott Laboratories, North Chicago IL, 1979.
12. Bergman HD: *Drug Intell Clin Pharm* 11: 345, 1977.
13. Trissel LA: *Parenteral Drug Information Guide*, Am Soc Hosp Pharm, Washington DC, 1974.
14. Trissel LA: *Handbook on Injectable Drugs*, 5th Ed, Am Soc Hosp Pharm, Washington DC, 1992.
15. Kobayashi NH, King JC: *Am J Hosp Pharm* 34: 589, 1977.
16. Sanders SJ et al: *Ibid* 35: 531, 1978.
17. Dudrick SJ, Rhoads JE: *Sci Am* 226: 73, 1972.

Bibliography

General

- Am J Hosp Pharm* 32: 261, 1975.
Avis, KE, Akers MJ: *Sterile Preparation for the Hospital Pharmacist*, Ann Arbor Sci Publ, Ann Arbor MI, 1981.

Trissel LA: *Handbook on Injectable Drugs*, 5th ed, Am Soc Hosp Pharm, Washington DC, 1992.

Turco SJ, King RE: *Sterile Dosage Forms: Their Preparation and Clinical Applications*, 3rd, Lea & Febiger, Philadelphia, 1987.

PCA

Buchanan C: Development of a Patient Controlled Analgesia Pump System. *Parenterals* 4: 2, 1986.

Graves DA *et al*: Morphine Requirements Using Patient-Controlled Analgesia; Influence of Diurnal Variation and Morbid obesity. *Clin Pharm* 2: 49, 1983.

White PF: Patient Controlled Analgesia: A New Approach to the Management of Postoperative Pain *Semin Anesth* 4: 255, 1985.

Williamson J *et al*: Implementation of an IV Morphine Infusion Program in a Community Hospital *Hosp Pharm* 21: 1098, 1986.

Implantable Systems

Ecoff E *et al*: Implantable Infusion Port, *NITA* 4: 406, 1983.

Fulks KD, Kenady DE: Techniques of Chemotherapy Delivery for Cancer Patients, *Hosp Formul* 22: 248, 1987.

Gyves J *et al*: Totally Implanted System for Intravenous Chemotherapy in Patients with Cancer, *Am J Med* 73: 841, 1983.

Kwan JW: Use Of Infusion Devices For Epidural Or Intrathecal Administration Of Spinal Opioids, *Am J Hosp Pharm* 47: 18, 1990.

May GS, Davis C: Percutaneous Catheters and Totally Implantable Access Systems, *J Intraven Nurs* 11: 97, 1988.

McGovern B *et al*: A Totally Implantable Venous Access System for Long Term Chemotherapy In Children, *J Pediatr Surg* 6: 725, 1985.

McIntyre KE *et al*: Early Experience with an Implantable Reservoir for Intravenous Chemotherapy, *Ariz Med* 42: 308, 1985.

CHA

Op

Geraik

Senior D
Alcon La
For Wor

Op:
free f
aged
includ
form:
aque
rolati
Op
divisi
clude
egory
prodi
revie
OTC
Th
prodi
tend
inten
larity
the p
more
ered
Pr
can't
eye:
uses
tivel
whit
clud
eyes
tiful
Fi
ogy
moc
that
solu
194
mer
in 1
thre
P
mer
hos
tion
the
oph
mo
C
uct
sur
off
opl
tioi
Ste
the
dat
pe:
Pri

Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein

(cytokine/soluble receptor/receptor family/lymphotoxin)

PATRICK W. GRAY, KATHY BARRETT, DAVID CHANTRY, MARTIN TURNER, AND MARC FELDMANN

Charing Cross Sunley Research Centre, Lurgan Avenue, Hammersmith, London, W6 8LW, England

Communicated by James Gowans, July 6, 1990

ABSTRACT The cDNA for one of the receptors for human tumor necrosis factor (TNF) has been isolated. This cDNA encodes a protein of 455 amino acids that is divided into an extracellular domain of 171 residues and a cytoplasmic domain of 221 residues. The extracellular domain has been engineered for expression in mammalian cells, and this recombinant derivative binds TNF α with high affinity and inhibits its cytotoxic activity *in vitro*. The TNF receptor exhibits similarity with a family of cell surface proteins that includes the nerve growth factor receptor, the human B-cell surface antigen CD40, and the rat T-cell surface antigen OX40. The TNF receptor contains four cysteine-rich subdomains in the extracellular portion. Mammalian cells transfected with the entire TNF receptor cDNA bind radiolabeled TNF α with an affinity of 2.5×10^{-9} M. This binding can be competitively inhibited with unlabeled TNF α or lymphotoxin (TNF β).

Tumor necrosis factor α (TNF α) is a potent cytokine that elicits a broad spectrum of biological responses. TNF α causes the cytolysis or cytostasis of many tumor cell lines *in vitro*, induces the hemorrhagic necrosis of transplanted tumors in mice, enhances the phagocytosis and cytotoxicity of polymorphonuclear neutrophils, and modulates the expression of many proteins, including lipoprotein lipase, class I antigens of the major histocompatibility complex, and cytokines such as interleukin 1 and interleukin 6 (1-4). TNF α appears to be necessary for a normal immune response (5, 6), but large quantities produce dramatic pathogenic effects (7-9). TNF α has been termed "cachectin" since it is the predominant factor responsible for the wasting syndrome (cachexia) associated with neoplastic disease and parasitemia (1, 3). TNF is also a major contributor to toxicity in Gram-negative sepsis, since antibodies against TNF can protect infected animals (7, 10).

The many activities of TNF α are mediated by binding to a cell surface receptor. Radioligand binding studies have confirmed the presence of TNF receptors on a wide variety of cell types (11-14). Although these receptors are expressed in limited numbers (1000-10,000 per cell), they bind TNF α with high affinity ($K_d = 10^9$ M $^{-1}$ at 4°C). The TNF receptor has been characterized as a 55- to 80-kDa glycoprotein that binds both TNF α and the structurally related lymphotoxin (TNF β). Lymphotoxin has biological activities that are similar, if not identical, to those of TNF α , presumably because both are recognized by the same receptor (4). Recently, several laboratories have detected heterogeneity in TNF receptor preparations (15, 16) and have proposed that at least two distinct cell surface molecules bind TNF α . In addition, both of these receptors appear to be released from cells in soluble form, as TNF-binding proteins of 30 kDa have been isolated from urine and serum (16-18). This soluble extracellular domain

retains the capacity to bind ligand with high affinity and therefore may be important in regulating concentrations of TNF α *in vivo*.

To further elaborate the structure of the TNF receptor, we have identified a cDNA for one of the receptor forms.* COS cells transfected with this cDNA bind TNF α with high affinity and this binding can be inhibited by unlabeled TNF α or lymphotoxin. A derivative of the TNF receptor, the extracellular domain, has also been expressed in COS cells. This results in the secretion of a soluble recombinant receptor domain with characteristics similar to those of the TNF-binding protein.

MATERIALS AND METHODS

Reagents. Recombinant human TNF α and TNF β were generously supplied by Genentech as highly purified proteins derived from *Escherichia coli*. The specific activities of these preparations were approximately 10^7 units/mg, as measured in the murine L929 cell cytotoxicity assay. The synthetic oligonucleotides were prepared by Oswel DNA Service (University of Edinburgh).

Isolation of TNF Receptor cDNA Clones. Purification and partial amino acid sequence analysis of the TNF-binding protein have been described (16, 17). The sequence of a peptide fragment (Glu-Met-Gly-Gln-Val-Glu-Ile-Ser-Ser-Thr-Val-Asp-Arg-Asp-Thr-Val-Cys-Gly) was used to design a synthetic oligodeoxynucleotide probe (5'-AAG-GAG-ATG-GGC-CAG-GTT-GAG-ATC-TCT-TCT-ACT-GTT-GAC-AAT-GAC-ACT-GTG-TGT-GGC-3'). The 57-mer DNA probe was labeled with 32 P by T4 polynucleotide kinase (New England Biolabs) and used to screen a placental cDNA library in λ gt10 (19, 20). Approximately 800,000 phage were transferred to nitrocellulose filters and screened at reduced stringency (21). Filters were incubated for 2 hr at 42°C in 0.05 M sodium phosphate, pH 6.5/20% formamide/0.75 M sodium chloride/0.075 M sodium citrate/1% polyvinylpyrrolidone (Sigma)/1% Ficoll/1% bovine serum albumin (Sigma), and sonicated salmon sperm DNA (Sigma) at 50 ng/ml. The radiolabeled probe was then added to the filters (10^6 cpm/ml), which were hybridized for 16 hr. Filters were washed extensively in 0.06 M sodium chloride/0.006 M sodium citrate/1% SDS at 37°C and positive clones were identified by autoradiography. Ten hybridizing clones were plaque-purified (19) and cDNA insert size was determined by polyacrylamide gel electrophoresis of *Eco*RI-digested phage DNA. The inserts of two cDNA clones were sequenced by the dideoxy chain-termination technique (22).

Southern and Northern Blot Analysis. DNA was isolated from human lymphocytes by the method of Blin and Stafford (23) and used for Southern blot analysis (24). DNA was

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TNF, tumor necrosis factor; PCR, polymerase chain reaction.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37764).

digested with restriction endonucleases (New England Biolabs), fractionated in a 1% agarose gel, and transferred to nitrocellulose. Hybridization and washing were conducted under stringent conditions (20) using a ^{32}P -labeled preparation of a 600-base-pair (bp) fragment of the TNF receptor cDNA. Northern blot analysis was performed (25) on oligo(dT)-selected RNA isolated from human placenta, spleen (generously provided by the Cooperative Human Tissue Network, Birmingham, AL), and fibroblast cell line 293. Following electrophoresis in a formaldehyde/1.2% agarose gel, the RNA was transferred to nitrocellulose and hybridized with the TNF receptor DNA probe under stringent conditions.

Mammalian Cell Expression of the Human TNF Receptor and Derivatives. The coding region of the majority of the human TNF receptor was isolated as an *EcoRI* fragment and cloned into a mammalian cell expression vector (26), resulting in plasmid pTNFR. The *EcoRI* fragment encodes 374 amino acids of the TNF receptor; 81 carboxyl-terminal residues of the cytoplasmic domain are therefore missing from this plasmid construction and 23 unrelated residues are added. A derivative of the TNF receptor was produced by engineering a termination codon just prior to the transmembrane domain (following Ile-159). The polymerase chain reaction (PCR) technique (27) was used to generate a 300-bp restriction fragment containing *Bgl* II site at the 5' end and a *HindIII* site preceded by a TAG stop codon at the 3' end. The PCR primers were 5'-GCTGCTCCAAATGCCGAAAG-3' and 5'-AGTTCAAGCTTTTACAGTGCCCTTAACATCTCTAA-3'. The PCR product was purified by gel electrophoresis and cloned into the TNF receptor expression plasmid (described above) digested with *Bgl* II and *HindIII*. DNA sequencing confirmed that the resulting plasmid (pTNFRecd) contained the designed DNA sequence.

The TNF receptor expression plasmids were transfected into monkey COS-7 cells by using Lipofectin (GIBCO/BRL) according to the manufacturer's instructions. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Analysis of Recombinant TNF Receptor Derivatives. TNF α was radioiodinated with the Iodo-Gen method (Pierce) according to the manufacturer's directions. The specific activity of the ^{125}I -labeled TNF α was 10–30 $\mu\text{Ci}/\mu\text{g}$ (1 μCi = 37 kBq). COS cells transfected with the TNF receptor cDNA (pTNFR, 1300-bp *EcoRI* fragment) were incubated for 24 hr and then seeded into six-well tissue culture plates (Nunc) at 4.5×10^5 cells per well. The cells were incubated for a further 48 hr and then receptor expression was quantitated by radioligand binding for 2 hr at 4°C. Nonspecific binding of ^{125}I -TNF α was determined in the presence of a 1000-fold molar excess of unlabeled TNF α . Binding data were analyzed by the method of Scatchard (28).

The TNF receptor derivative was analyzed for inhibition of ^{125}I -TNF α binding to the natural receptor on human U-937 monocytic cells. Culture supernatant was harvested 72 hr after COS cells were transfected with pTNFRecd. U-937 cells (2×10^6 cells in 200 μl) were incubated with 1 nM ^{125}I -TNF α and dilutions of COS cell medium for 2 hr at 4°C. Cells were then centrifuged through 20% sucrose to remove unbound TNF α . Nonspecific binding was determined in the presence of 1 μM unlabeled TNF α .

The TNF receptor derivative was also analyzed for inhibition of TNF α cytotoxic effects *in vitro*. The cytotoxicity assay was performed as described on the TNF-sensitive cell line WEHI 164 clone 13 (29). Serial dilutions of culture supernatant from COS cells transfected with pTNFRecd or from mock-transfected controls were incubated with a constant amount of TNF α (1 ng/ml) for 1 hr at 37°C before addition to the assay.

RESULTS

Isolation and Characterization of the TNF Receptor cDNA. Partial amino acid sequence of the TNF-binding protein (16, 17) was used to design a synthetic oligonucleotide probe. The radiolabeled probe was used to screen a human placental cDNA library in $\lambda\text{gt}10$, and 10 hybridizing phage were isolated. The nucleotide and deduced amino acid sequences of the longest cDNA clone are depicted in Fig. 1. The third potential ATG initiation codon occurs at position 156 of the nucleotide sequence; the first two ATG codons are closely followed by termination codons, and the third ATG is preceded by the best translation initiation consensus nucleotides (30). The cDNA encodes an open reading frame of 1365 bases that codes for a polypeptide of 455 residues. The peptide sequence determined by amino acid sequencing was identified in the encoded cDNA (18 of 19 matching residues). The amino-terminal end identified for the TNF-binding protein corresponds to the cDNA-encoded sequence (17 of 19 matching residues) beginning at residue 41. The first 35 amino acids are generally quite hydrophobic and probably represent a signal sequence. Residues 36–40 (–5 to –1 in Fig. 1) are highly charged (Asp-Arg-Glu-Lys-Arg) and such a sequence is not typically found in secretory signal sequences (31); perhaps the natural receptor is processed by proteolysis after residues 39 and 40, which comprise a dibasic cleavage site (Lys-Arg). Hydropathy analysis of the protein sequence predicts a signal transmembrane domain of 23 amino acids. This hydrophobic sequence divides the protein into an extracellular domain of 171 residues and a cytoplasmic domain of 221 residues. The amino acid composition determined for the TNF-binding protein (17) corresponds well with the predicted composition of the extracellular domain encoded by the cDNA (results not shown). The discrepancy between the predicted receptor size (49 kDa) and the size determined by SDS/polyacrylamide gel electrophoresis (55–60 kDa, refs. 12–14) is probably due to glycosylation; there are four potential N-linked glycosylation sites in the sequence, three of which are in the extracellular domain. The sequence contains a large number (i.e., 30) of cysteine residues, 24 of which are in the extracellular domain. The arrangement of these cysteines is similar to that of several other cell surface proteins (see *Discussion*), suggesting that the TNF receptor is structurally related to a family of receptors.

A Northern blot analysis is presented in Fig. 2A. The ^{32}P -labeled 600-bp cDNA fragment hybridized to a single predominant band of oligo(dT)-selected RNA from human placenta or spleen. A minor larger transcript was also observed in RNA from a fibroblast cell line. The size of the hybridizing species is 2400 bases, in good agreement with the size of isolated cDNA. Fig. 2B shows a Southern blot of human genomic DNA hybridized with the same 600-bp probe from the cDNA. In each of the three different restriction digests, only a single hybridization signal was observed. Thus the TNF receptor that we have isolated appears to be encoded by a single gene.

Expression of Recombinant TNF Receptor Sequences in Mammalian Cells. To confirm that the cDNA shown in Fig. 1 indeed encodes the TNF receptor, the cDNA was engineered for expression in mammalian cells. The cDNA contains an *EcoRI* site at position 1270 of Fig. 1. The receptor coding sequence was isolated as a 1300-bp *EcoRI* fragment (containing all but the last 81 codons of the cytoplasmic domain) and inserted into the mammalian cell expression vector containing a cytomegalovirus promoter and simian virus 40 transcription termination sequences (26). The resulting plasmid was transfected into COS cells, which were analyzed for TNF receptor expression after 3 days. As shown in Fig. 3A, the transfected cells specifically bound radioiodinated TNF α in a saturable and dose-dependent fashion. The

```

1  ACCA GTGATCTCTA TGCCCGAGTC TCAACCTCA ACTGTCACCC CAAGGCAC TT GGCAGTCCT GGACAGACCG
75  AGTCCCGGCA AGCCCGGCA CTGCGCTGC CACACTGCCG TGAGCCCAAA TGCGGGAGTG AGAGGCCATA GCTGCTGGC

-40 M G L S T V P D L L L P L V L L L E L L V G I Y P
156 ATG GGC CTC TCC ACC GTG CCT GAC CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC

-16 S G V I G L V P H L G D R E K R Y D S V C P O G K
228 TCA GGG GTT ATT GGA CTG GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA

9 Y I H P O N N S I C C T K C H K G T Y L Y N D C
300 TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT

33 P G P G O D T D C R E C E S G S F T A S E N H L
372 CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC GCT TCA GAA AAC CAG CTC

57 R H C L S C S K C R K E M G O V E I S S C T V D
444 AGA CAC TGC CTC GAG TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC

81 R D T V C G C R K N Q Y R H Y W S E N L F Q C F
516 CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC

125 N C S L C L N G T V H L S C O E K O N T V C T C
558 AAT TGC AGC CTC TGC CTC AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC

229 H A G F F L R E N E C V S C S N C K K S L E C T
600 CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG

153 K L C L P O I E N V K G T E D S G T T V L L P L
722 AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC ACA GTG CTG TTG CCC CTG

177 V I F F G L C L L S L L F I G L M Y R Y Q R W K
804 GTC ATT TTC TTT GGT CTT TTA TCC CTC CTC TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG

201 S K L Y S I V C G K X S T P E K E G E L E G T T T
876 TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TGC ACA CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT ACT ACT

225 K P L A P N P S F S P T P G F T P T L G F S P V
948 AAG CCC CTG GGC CCA AAC CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC CCC ACC CTG GGC TTC AGT CCC GTG

249 P S S T F T S S S T Y T P G D C P N F A A P R R
1020 CCC AGT TCC ACC TCC AGC TCC ACC TAT ACC CCC GGT GAC TGT CCC AAC TTT GCG GGT CCC CGC AGA

273 E V A P P Y Q G A D P I L A T A L A S D P I P N
1092 GAG GTG GCA CCA CCC TAT CAG GGG GCT GAC CCC ATC CTT GCG ACA GCC CTC GGC TCC GAC CCC ATC CCC AAC

297 P L Q K W E D S A H K P Q S L D T D O P A T L Y
1164 CCC CTT CAG AAG TGG GAG GAC AGT GGC CAC AAG CCA CAG AGC CTA GAC ACT GAT GAC CCC GCG AGC GTG TAC

321 A V V E N V P P L R T L E F V R R L G L S D H E
1236 GGC GTG GTG GAG AAC GTG CCC CCG TTG CGC TGG AAG GAA TTC GTG CGG CGC CTA GGS CTG AGC GAC CAC GAG

345 I D R L E L Q N G R C L R E A O Y S M L A T W R
1308 ATC GAT CGG CTG GAG CTG CAG AAC GGG CGC TGC CTG CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC TGG AGG

369 R T P R R E L L G R V L R N M D L L G
1380 CGG CGC AGC CGG CGG CGC GAG GCC ACG CTG GAG CTG CTG GGA CGC GTG CTC CGC GAC ATG GAC CTG CTG GGC

393 C L E D I E E A L C G P A A L P P A P S L L R
1452 TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC GGC CCC GGC GCG CTC CCG CCC GCG CCC AGT CTT CTC AGA TGA

1521 GGC TGG GGC TGG GGG GAG CTT AAG GAC GTG CCG GAG ATG CCG CTT CCA ACG CCG CTT TTT TCT GGA AGG AGG GTC

1601 CTG CAG GGC AAG CAG GAG TAG CAG CCG CTA CTT GGT GAG CTA ACG CCG CTA GAT GAT CTA GCT TTT CTA GCT GCG TGC

1681 CCG CCG GAG AGT CAG GGT GTG CCG GCG AGAGAGTGC GCG TGG GCT CAAGAGCTG AGTGGTGTG TTGCGAGAT

1761 GAGGAGGCT ATGCTCATG CCGGTTTGG GTGCTCTAC CAGCAAGGCT GCTCGGGGC CCGTGGTTCG TCCGAGGCC

1841 TTTTTCAG TGCATAAGCA GTTTTGTG TTTTGTGTTT GTTTTAA TCAATCATGT TACACTAATA

1921 GAAATGTGG ACTCTGTGC CTTCTGCTG GACAAGCAC ATAGCAAGCT GAATGTCTT AAGGCGGGG CGAGCAGCGA

2001 ACAATGGGG CTTCAGTGG AGCTGTGGAC TTTTGTACAT ACATAAAAT TCTGAAGTTA AG

```

FIG. 1. Nucleotide sequence of the human TNF receptor cDNA and encoded amino acid sequence. The predicted signal sequence is numbered -40 to -1. The first residue of the TNF-binding protein (16, 17) is preceded by an arrowhead, the transmembrane domain is boxed, and potential N-linked glycosylation sites in the extracellular domain are overlined. The sequence homologous with the designed oligonucleotide probe is found at nucleotide positions 477-533.

population of COS cells expressed $\approx 1 \times 10^5$ receptors per cell. The measured binding affinity of recombinant receptors was $2.5 \times 10^9 \text{ M}^{-1}$ at 4°C , which is in close agreement with the value for the natural receptor on human cells (13, 14). The binding of ^{125}I -TNF α (1 nM) to these cells could be inhibited by the addition of unlabeled TNF α or lymphotoxin (Fig. 3B). COS cells transfected with just the expression vector did not significantly bind ^{125}I -TNF α (<2% of the binding seen with the cDNA transfection).

The extracellular domain of the TNF receptor is naturally shed from cells (16-18). To produce a similar recombinant derivative, at stop codon preceding the transmembrane domain was engineered into the cDNA by PCR mutagenesis. The modified DNA was inserted into the expression plasmid and subsequently transfected into COS cells. After 3 days, the COS cell medium was tested for inhibition of TNF α binding to human U-937 cells. As shown in Fig. 4A, the transfected-cell medium inhibited $\approx 70\%$ of the binding of TNF α . The recombinant TNF receptor derivative was next tested for inhibition of TNF α biological activity. A sensitive bioassay for TNF α is measurement of cytotoxicity of mouse WEHI 164 (clone 13) cells. The transfected-cell medium inhibited 60% of TNF α cytotoxicity on this cell line (Fig. 4B).

Medium from mock-transfected COS cells did not inhibit TNF α cytotoxicity or binding. These experiments demonstrate that the recombinant extracellular domain of the TNF receptor is capable of binding TNF and inhibiting its biological activity.

DISCUSSION

This paper describes the cDNA cloning of a human TNF receptor. The cDNA was isolated with a synthetic oligonucleotide probe based on partial amino acid sequence. The two peptide sequences determined for the purified TNF-binding protein correspond to sequences encoded in the cDNA. Confirmation that this cDNA encodes the TNF receptor was established by mammalian cell expression studies: this cDNA directs the expression of a cell surface protein that specifically binds TNF α with high affinity.

Southern hybridization suggests that the cDNA is encoded by a single gene. Several other laboratories have provided strong evidence for at least two structurally distinct human TNF receptors (15, 16). Consequently, other forms of the TNF receptor must differ significantly from that isolated here, since only single hybridization signals are observed in

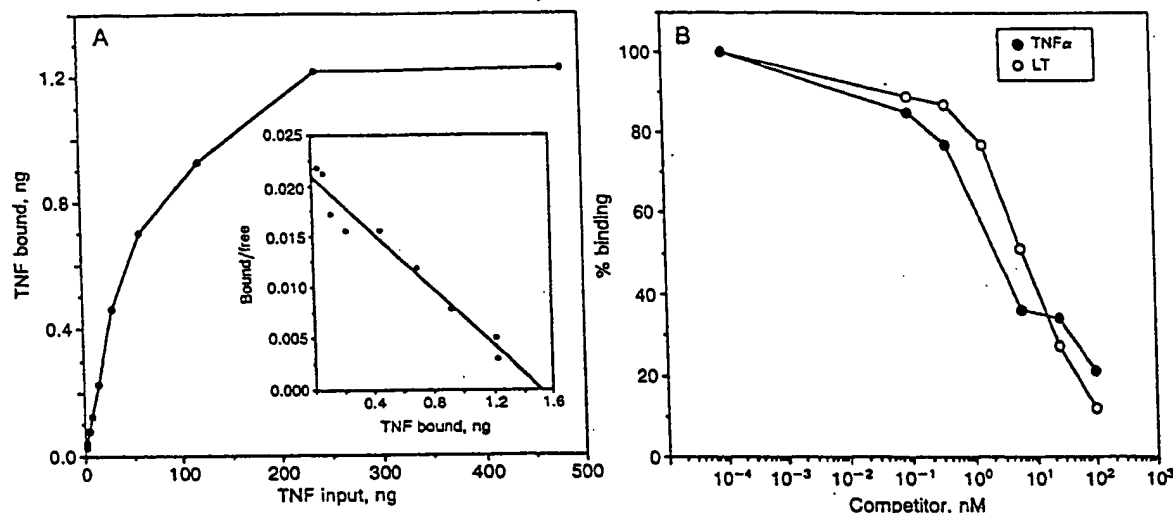


FIG. 3. Binding characteristics of recombinant human TNF receptor expressed in COS-7 cells. (A) Direct binding of recombinant ^{125}I -TNF α to COS-7 cells transfected with pTNFR. (Inset) Scatchard analysis derived from these data. (B) Competition analysis. Monolayers of COS-7 cells transfected with the TNF receptor cDNA were incubated with 1 nM ^{125}I -TNF in the presence of various concentrations of unlabeled TNF α (●) or TNF β (lymphotoxin, LT) (○).

the Southern analysis (Fig. 2B). Similarly, the ligands for this receptor, TNF α and lymphotoxin, share 35% amino acid similarity but their coding sequences do not cross-hybridize (32).

The TNF receptor exhibits significant amino acid sequence homology to three other cell surface proteins: the low-affinity nerve growth factor receptor (28% similarity; ref. 33), human CD40 (a B-cell surface marker; 25% similarity, ref. 34), and rat OX40 (found on activated T cells that are CD4-positive; 24% identity; ref. 35). The similarity among these proteins is confined to the extracellular domain. As shown in Fig. 5, each of these contain four (three in OX40) cysteine-rich subdomains that probably evolved from a common motif. Consequently, these proteins are members of a family of cell surface molecules that are structurally related. The distinct functions of each of these molecules may be a result of their dissimilar cytoplasmic domains. The ligands for OX40 and CD40 have not been identified, but antibodies against them

can augment T-cell and B-cell responses, respectively (34, 35). TNF α and nerve growth factor do not share sequence similarity, but both ligands affect the growth and differentiation of target cells. Perhaps this family of cell surface molecules has evolved to recognize structurally distinct ligands but retained a common scaffold that is generally useful for recognition of polypeptide ligands. While the dissimilar cytoplasmic domains suggest independent modes of signal transduction, each receptor appears to function in the growth or differentiation of the cell.

The natural production of a soluble receptor domain has been observed for other cytokine receptors, including those for interleukins 2, 4, and 6 and interferon γ (36). The formation of a soluble extracellular domain can arise by several mechanisms—for example, by degradation of the receptor (as is the case for the interleukin 2 receptor) or by synthesis of an independent transcript that does not encode a transmembrane domain [as seen for the interleukin 4 receptor (37) and the soluble or cell-bound forms of immu-

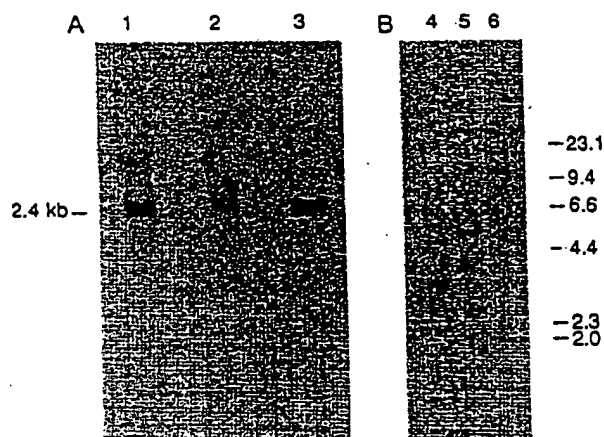


FIG. 2. (A) Northern blot of oligo(dT)-selected RNA (10 μg per lane) from human 293 cells (fibroblast cell line) (lane 1), placenta (lane 2), and spleen (lane 3) hybridized with the TNF receptor cDNA (*Sma* I-*Eco*RI fragment). kb, Kilobases. (B) Southern blot of human genomic DNA (5 μg per lane) digested with *Pst* I (lane 4), *Hind*III (lane 5), or *Eco*RI (lane 6) and hybridized with the same probe as used for the Northern blot.

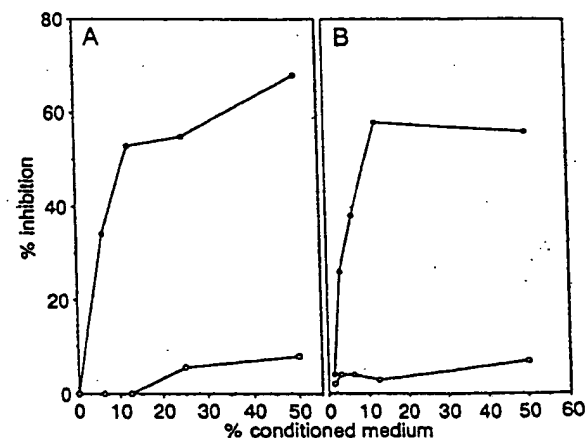


FIG. 4. Effects of soluble TNF receptor on TNF binding and biological activity. (A) Effect of culture supernatants from COS-7 cells transfected with a cDNA encoding a soluble form of the TNF receptor (pTNFRcd; ●) or mock-transfected (○) on ^{125}I -TNF binding to U-937 cells. (B) Effect of these supernatants on TNF-mediated killing of WEHI 164 (clone 13) line.

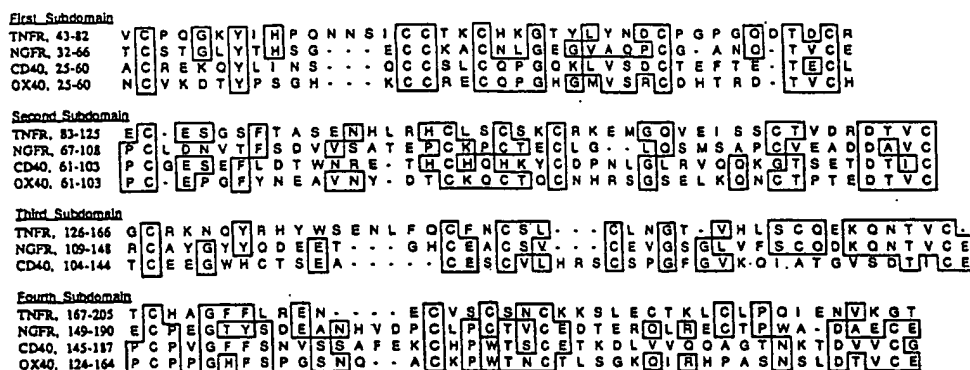


FIG. 5. Alignment of the cysteine-rich subdomains of the extracellular portions of the human TNF receptor (TNFR), rat nerve growth factor receptor (NGFR; ref. 33), human CD40 (34), and rat OX40 (35). Common residues are boxed. OX40 contains only three subdomains and lacks the third. Residue numbers refer to the precursor form and begin with the initiating methionine.

noglobulin]. We observed a single predominant signal in a Northern blot of human spleen and placenta mRNA, suggesting that one size of transcript produces this form of the TNF receptor. Consequently, the formation of the TNF-binding protein is most likely a result of proteolytic cleavage from the membrane-bound receptor. Presumably the extracellular domain is cleaved by proteolysis near the transmembrane junction, resulting in the release of a soluble receptor fragment.

By introducing a termination codon prior to the transmembrane domain, we have expressed a soluble form of the extracellular domain. This recombinant product mimics the natural TNF-binding protein in its sequence, amino acid composition, and ability to inhibit TNF biological activity (16, 17). The natural TNF-binding protein may play an important role in the regulation of TNF-mediated responses by binding and sequestering the cytokine. The recombinant extracellular domain may similarly provide therapeutic benefit in disorders such as cachexia, sepsis, and autoimmune diseases where TNF has been shown to play a significant causative role, such as rheumatoid arthritis (38). Attempts to demonstrate the therapeutic potential of the recombinant soluble TNF receptor can now be instigated.

Note. After submission of this manuscript for review, two papers appeared (39, 40) describing the sequence of the human TNF receptor cDNA, which is identical to that reported here.

We thank Anne Hales, Peter Katsikis, and Majja Kissonerghis for their expert technical assistance and Brian Foxwell for helpful discussions. This work was supported by the Medical Research Council (England), the Arthritis and Rheumatism Council, the U.S. Navy Medical Research and Development Command, Xenova Ltd., and the Sunley Trust.

- Beutler, B. & Cerami, A. (1989) *Annu. Rev. Immunol.* 7, 625-655.
- Old, L. J. (1988) *Sci. Am.* 258, 41-49.
- Beutler, B. & Cerami, A. (1988) *Annu. Rev. Biochem.* 57, 505-518.
- Goeddel, D. V., Aggarwal, B. B., Gray, P. W., Nedwin, G. E., Palladino, M. A., Patton, J. S., Pennica, D., Shepard, H. M., Sugarman, B. J. & Wong, G. H. W. (1986) *Cold Spring Harbor Symp. Quant. Biol.* 51, 597-609.
- Kindler, V., Sappino, A.-P., Grau, G. E., Pigeot, P.-F. & Vassalli, P. (1989) *Cell* 56, 731-740.
- Shalaby, M. R., Espevik, T., Rice, G. C., Ammann, A. J., Figari, I. S., Ranges, G. E. & Palladino, M. A., Jr. (1988) *J. Immunol.* 141, 499-503.
- Tracey, K. J., Beutler, B., Lowry, S. F., Merryweather, J., Wolpe, S., Milsark, I. W., Hariri, R. J., Fahey, T. J., III, Zentella, A., Alpert, J. D., Shires, T. & Cerami, A. (1986) *Science* 234, 470-473.
- Gray, G. E., Fajardo, L. F., Piquet, P.-F., Lambert, P.-H. & Vassalli, P. (1987) *Science* 237, 1210-1213.

- Piquet, P. F., Collart, M. A., Grau, G. E., Kapanci, Y. & Vassalli, P. (1989) *J. Exp. Med.* 170, 655-663.
- Tracey, K. J., Fong, Y., Hesse, D. G., Manogue, K. R., Lee, A. T., Kuo, G., Lowry, S. F. & Cerami, A. (1987) *Nature (London)* 330, 662-664.
- Aggarwal, B. B., Essalu, T. E. & Hass, P. E. (1985) *Nature (London)* 318, 665-667.
- Creasy, A. A., Yamamoto, R. & Vitt, C. R. (1987) *Proc. Natl. Acad. Sci. USA* 84, 3293-3297.
- Stauber, G. B., Aiyer, R. A. & Aggarwal, B. B. (1988) *J. Biol. Chem.* 263, 19098-19104.
- Scheurich, P., Ucer, U., Kronke, M. & Pfitzenmaier, K. (1986) *Int. J. Cancer* 38, 127-133.
- Hohmann, H., Remy, R., Brockhaus, M. & van Loon, P. G. M. (1989) *J. Biol. Chem.* 264, 14927-14934.
- Englemann, H., Novick, D. & Wallach, D. (1990) *J. Biol. Chem.* 265, 1531-1536.
- Olsson, I., Lantz, M., Nilsson, E., Peetre, C., Thysell, H., Grubb, A. & Adolf, G. (1989) *Eur. J. Haematol.* 42, 270-275.
- Seckinger, P., Isaacs, S. & Dayer, J.-M. (1989) *J. Biol. Chem.* 264, 11966-11973.
- Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, G. K. & Efstratiadis, A. (1978) *Cell* 15, 687-701.
- Lawn, R. M., Fritsch, E. F., Parker, R. C., Blake, G. & Maniatis, T. (1978) *Cell* 15, 1157-1174.
- Gray, P. W., Leong, S. R., Fennie, E., Farrar, M. A., Pingel, J. T. & Schreiber, R. D. (1989) *Proc. Natl. Acad. Sci. USA* 86, 8497-8501.
- Smith, A. J. H. (1980) *Methods Enzymol.* 65, 560-580.
- Blin, N. & Stafford, D. W. (1976) *Nucleic Acids Res.* 3, 2303-2308.
- Southern, E. M. (1975) *J. Mol. Biol.* 98, 503-517.
- Dobner, P. R., Kawasaki, E. S., Yu, L. Y. & Bancroft, F. C. (1981) *Proc. Natl. Acad. Sci. USA* 78, 2230-2234.
- Eaton, D. L., Wood, W. I., Eaton, D., Hass, P. E., Hollingshead, P., Wion, K., Mather, J., Lawn, R. M., Vohar, G. A. & Gorman, C. (1986) *Biochemistry* 25, 8343-8347.
- Scharf, S. J., Horn, G. T. & Erlich, H. A. (1986) *Science* 233, 1076-1079.
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* 51, 660-672.
- Espevik, T. & Nissen-Meyer, J. (1986) *J. Immunol. Methods* 95, 99-105.
- Kozak, M. (1989) *J. Cell Biol.* 108, 229-241.
- von Heijne, G. (1986) *Nucleic Acids Res.* 14, 4683-4690.
- Nedwin, G. E., Naylor, S. L., Sakaguchi, A. Y., Smith, D., Jarrett-Nedwin, J., Pennica, D., Goeddel, D. V. & Gray, P. W. (1985) *Nucleic Acids Res.* 13, 6361-6373.
- Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A. & Shooter, E. M. (1987) *Nature (London)* 325, 593-597.
- Stamenkovic, I., Clark, E. A. & Seed, B. (1989) *EMBO J.* 8, 1403-1410.
- Mallett, S., Fossum, S. & Barclay, A. N. (1990) *EMBO J.* 9, 1063-1068.
- Novick, D., Englemann, H., Wallach, D. & Rubinstein, M. (1989) *J. Exp. Med.* 170, 1409-1414.
- Mosley, B., Beckmann, M. P., March, C. J., Idzerda, R. L., Gimpel, S. D., Van den Bos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., Wignall, J. M., Smith, C., Gallis, B., Sims, J. E., Urdal, D., Widmer, M. B., Cosman, D. & Park, L. S. (1989) *Cell* 59, 335-348.
- Brennan, F. M., Chantry, D., Jackson, A., Maini, R. N. & Feldmann, M. (1989) *Lancet* ii, 244-247.
- Loetschere, H., Pan, Y.-C. E., Lahm, H.-W., Gentz, R., Brockhaus, M., Tabuchi, H. & Lesslauer, W. (1990) *Cell* 61, 351-359.
- Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H. W., Gatanaga, T., Granger, G. A., Lentz, R., Raab, H., Kohr, W. J. & Goeddel, D. V. (1990) *Cell* 61, 361-370.

Protection against endotoxic shock by a tumor necrosis factor receptor immunoadhesin

(septic shock/cachectin/lymphotoxin/immunoglobulin chimera)

AVI ASHKENAZI^{*†}, SCOT A. MARSTERS^{*}, DANIEL J. CAPON^{*‡}, STEVEN M. CHAMOW[§], IRENÉ S. FIGARI[¶],
DIANE PENNICA^{||}, DAVID V. GOEDDEL^{||}, MICHAEL A. PALLADINO[¶], AND DOUGLAS H. SMITH^{*‡}

Departments of ^{*}Immunobiology, [§]Process Science, [‡]Cell Biology, and [¶]Molecular Biology, Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080

Communicated by David R. Davies, August 19, 1991 (received for review June 13, 1991)

ABSTRACT Tumor necrosis factors (TNF) α and β are structurally related cytokines that mediate a wide range of immunological, inflammatory, and cytotoxic effects. During bacterial infection of the bloodstream (sepsis), TNF- α induction by bacterial endotoxin is thought to be a major factor contributing to the cardiovascular collapse and critical organ failure that can develop. Despite antibiotic therapy, these consequences of sepsis continue to have a high mortality rate in humans. Here we describe a potent TNF antagonist, a TNF receptor (TNFR) immunoadhesin, constructed by gene fusion of the extracellular portion of human type 1 TNFR with the constant domains of human IgG heavy chain (TNFR-IgG). When expressed in transfected human cells, TNFR-IgG is secreted as a disulfide-bonded homodimer. Purified TNFR-IgG binds to both TNF- α and TNF- β and exhibits 6- to 8-fold higher affinity for TNF- α than cell surface or soluble TNF receptors. *In vitro*, TNFR-IgG blocks completely the cytolytic effect of TNF- α or TNF- β on actinomycin D-treated cells and is markedly more efficient than soluble TNFR (24-fold) or monoclonal anti-TNF- α antibodies (4-fold) in inhibiting TNF- α . *In vitro*, TNFR-IgG prevents endotoxin-induced lethality in mice when given 0.5 hr prior to endotoxin and provides significant protection when given up to 1 hr after endotoxin challenge. These results confirm the importance of TNF- α in the pathogenesis of septic shock and suggest a clinical potential for TNFR-IgG as a preventive and therapeutic treatment in sepsis.

Tumor necrosis factors α (TNF- α ; cachectin) and β (TNF- β ; lymphotoxin) are related proteins, secreted by activated macrophages and lymphocytes, respectively (1–3). These cytokines have been implicated in diverse biological processes including immunoregulation, inflammation, antiviral defense, cachexia, angiogenesis, and septic shock. The biological effects of TNF- α and TNF- β are mediated through specific receptors. Molecular cloning has demonstrated the existence of two distinct types of TNF receptor (TNFR), each of which binds to both TNF- α and TNF- β (4–8). The extracellular portions of both receptors are found naturally also as soluble TNF binding proteins (7, 8).

Several lines of evidence indicate that TNF- α is a principal mediator in the pathogenesis of septic shock. First, neutralizing anti-TNF- α antibodies can prevent the pulmonary failure and death associated with administration of endotoxin or *Escherichia coli* in mice (9) or baboons (10). Second, intravenous infusion of TNF- α leads to a toxic syndrome indistinguishable from that caused by endotoxemia and gram-negative sepsis (11, 12). In addition, the levels of TNF- α increase substantially in the circulation of animals and hu-

mans who have received endotoxin or have septic shock (13, 14) and correlate with mortality in severe sepsis (15–17).

To create a TNF antagonist that might block the lethal effect of TNF in endotoxic shock, we constructed an immunoadhesin (18) containing the extracellular portion of human type 1 TNFR and the hinge and Fc regions of human IgG heavy chain (TNFR-IgG). This approach was based on the observation that the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), can be linked to IgG heavy chain, thus creating a protein with two functional HIV binding sites and a markedly longer plasma half life than the soluble extracellular portion of CD4 (18, 19). We show that the TNFR-IgG chimera acts as a potent antagonist of TNF- α and TNF- β *in vitro* and can prevent endotoxin-induced lethality in a mouse model for septic shock. These results suggest that TNFR-IgG may be useful against the potentially lethal consequences of sepsis in humans.

MATERIALS AND METHODS

Construction, Expression, and Purification of TNFR-IgG. The schematic structure of TNFR-IgG is shown in Fig. 1A. A mammalian expression vector encoding TNFR-IgG (pRK-TNFR-IgG) was constructed from plasmids encoding the human type 1 TNFR (pRK-TNF-R) (4) and CD4-IgG (pRKCD4₂Fc₁) (19). A 770-base-pair (bp) DNA fragment containing 5' untranslated sequences and encoding the leader and extracellular portion of type 1 TNFR was generated by digesting pRK-TNF-R with *EcoRI* and *HindIII*. Plasmid pRKCD4₂Fc₁ encoding the extracellular domain of CD4 fused to the hinge and Fc region of human IgG₁ heavy chain (19), was digested with *EcoRI* and *NdeI* to remove most of the CD4 sequence while retaining the IgG₁ sequence. The TNFR-encoding fragment was then inserted 5' of the IgG₁ sequence and in the same reading orientation by ligating the respective *EcoRI* sites and by blunting and ligating the *HindIII* and *NdeI* sites. The remaining CD4 sequence was removed to create the exact junction between threonine-171 of TNFR and aspartic acid-216 of IgG₁ heavy chain by oligonucleotide-directed deletional mutagenesis, using synthetic oligonucleotides complementary to the 24 nucleotides at the borders of the desired TNFR, and IgG₁ fusion sites as primers and the plasmid described above as a template (18). The final DNA construct was sequenced to confirm the correct primary structure. The mature TNFR-IgG polypeptide encoded by pRKTNFR-IgG thus contains 171 residues from TNFR and 227 residues from IgG₁—i.e., a total of 398

Abbreviations: TNF, tumor necrosis factor; TNFR, TNF receptor; C_H, heavy-chain constant region; V_H, heavy-chain variable region; TNFR-IgG, fusion of TNFR with IgG₁ heavy-chain hinge region and C_H2 and C_H3 domains; HIV, human immunodeficiency virus; sTNFR, soluble TNFR.

[†]To whom reprint requests should be addressed.

[‡]Present address: Cell Genesys, Inc., 344 Lakeside Drive, Foster City, CA 94040.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

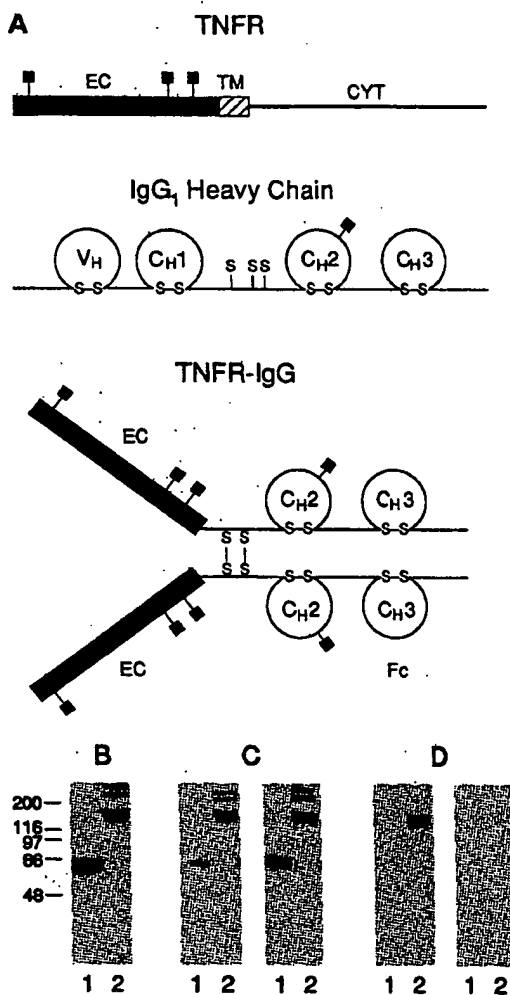


FIG. 1. (A) Schematic structure of the human type 1 TNFR, human IgG₁ heavy chain, and TNFR-IgG. The extracellular (EC), transmembrane (TM), and cytoplasmic (CYT) domains of TNFR and the IgG₁ heavy-chain variable region (V_H) and constant region (domains C_H1, C_H2, and C_H3) are indicated. TNFR-IgG was constructed by gene fusion of the extracellular domain of TNFR with the hinge region and C_H2 and C_H3 domains of the IgG₁ heavy chain. Potential asparagine-linked glycosylation sites (■) and disulfide bonds (S-S) (IgG protein only) are shown also. (B-D) Subunit structure and functional domains of TNFR-IgG. Human embryonic kidney 293 cells were transfected with a vector directing transient expression of TNFR-IgG. The protein was recovered from culture supernatants and purified by affinity chromatography on *S. aureus* protein A. SDS/polyacrylamide gel electrophoresis was carried out under reducing (lane 1) or nonreducing (lane 2) conditions. The proteins were stained with Coomassie blue (B) or electroblotted onto nitrocellulose paper and incubated with antibodies to human TNFR (C Left) or human IgG Fc (C Right) or with ¹²⁵I-TNF- α (1 nM) in the absence (D Left) or presence (D Right) of unlabeled TNF- α (100 nM). Blots were developed with horseradish peroxidase-conjugated second antibody (C) or autoradiography (D).

amino acids. TNFR-IgG was expressed in human embryonic kidney 293 cells by transient transfection with pRKTNFR-IgG by the calcium phosphate precipitation method as described (18). TNFR-IgG was purified to >95% homogeneity from serum-free cell culture supernatants by affinity chromatography on *Staphylococcus aureus* protein A. TNFR-IgG was eluted with 50 mM sodium citrate, pH 3/20% (wt/vol) glycerol, and the pH was neutralized with 0.05 vol of 3 M Tris-HCl (pH 8-9).

TNF Binding Assays. Binding of TNFR-IgG to TNF was analyzed essentially as described for CD4-IgG binding to HIV gp120 (20). TNFR-IgG (1 μ g/ml) was immobilized onto microtiter wells coated with goat anti-human IgG Fc antibody. Reactions with recombinant human ¹²⁵I-TNF- α (radioiodinated by using lactoperoxidase to a specific activity of 19.1 μ Ci/ μ g; 1 μ Ci = 37 kBq) were done in phosphate-buffered saline (PBS) containing 1% bovine serum albumin for 1 hr at 24°C. Nonspecific binding was determined by omitting TNFR-IgG. In competition binding analyses, ¹²⁵I-TNF- α was incubated with immobilized TNFR-IgG in the presence of increasing concentrations of unlabeled TNF. The K_d was determined from competition IC₅₀ values according to the following equation: $K_d = IC_{50}/(1 + [T]/K_{dT})$, where [T] is the concentration of the tracer (0.1 nM) and K_{dT} is the K_d of the tracer determined by saturation binding (80 pM).

TNF Cytotoxicity Assays. TNF cytotoxicity was assayed essentially as described (21). Murine L-M cells were plated in microtiter dishes (4 \times 10⁴ cells per well) and treated with actinomycin D (3 μ g/ml) and TNF- α or TNF- β (1 ng/ml) in the absence or presence of TNFR-IgG or other inhibitors. After 20 hr of incubation at 39°C, the cell survival was determined by a crystal violet dye exclusion test.

Mouse Model for Septic Shock. Septic shock was modeled by endotoxin injection of 6- to 8-week-old female BALB/c mice. Animals were injected intravenously (i.v.) with an LD₁₀₀ dose of *Salmonella abortus*-derived endotoxin (175 μ g per mouse) in phosphate-buffered saline (PBS), and survival was followed for at least 78 hr. Purified TNFR-IgG, or CD4-IgG used as a negative control, were diluted in PBS and injected i.v. prior to or after endotoxin administration.

RESULTS

Subunit Structure of TNFR-IgG. TNFR-IgG was created by fusing complementary DNAs encoding the extracellular portion (amino acids 1-171) of human 55-kDa type 1 TNFR and the hinge region and constant region C_H2 and C_H3 domains (amino acids 216-443) of human IgG₁ heavy chain (Fig. 1A). A vector directing mammalian expression of TNFR-IgG was introduced transiently into human kidney 293 cells to produce the molecule as a secreted protein. Taking advantage of the presence of an IgG Fc domain in TNFR-IgG, we used protein A affinity chromatography to recover and purify the protein from cell culture supernatants. We examined the subunit structure of TNFR-IgG by SDS/polyacrylamide gel electrophoresis (Fig. 1B-D). Under reducing conditions, a molecular mass of \approx 60 kDa was observed, whereas under nonreducing conditions, it was approximately doubled, indicating that TNFR-IgG is a disulfide-bonded dimer (Fig. 1B). Minor bands of higher molecular mass were observed also, suggesting some aggregation of TNFR-IgG. Western blot analyses showed reactivity of TNFR-IgG with antibody to the type 1 human TNFR or to human IgG Fc (Fig. 1C) and showed specific binding of human ¹²⁵I-TNF- α (Fig. 1D). Notably, ¹²⁵I-TNF- α did not bind to reduced TNFR-IgG, suggesting that intramolecular disulfide bonds in TNFR are required for binding to TNF- α . These results indicate a covalent homodimeric structure for TNFR-IgG and the presence of functional TNF binding and antibody Fc domains in this protein.

Binding of TNFR-IgG to TNF- α and TNF- β . To investigate the binding of TNFR-IgG to TNF- α and TNF- β , we carried out saturation and competition binding analyses, using an assay in which TNFR-IgG was immobilized by binding of its Fc domain to anti-Fc antibodies coated on microtiter wells. Human ¹²⁵I-TNF- α bound to a single class of sites with an apparent dissociation constant (K_d) of 80 ± 20 pM (Fig. 2 Left). Human TNF- β was able to displace the binding of ¹²⁵I-TNF- α completely, confirming previous observations

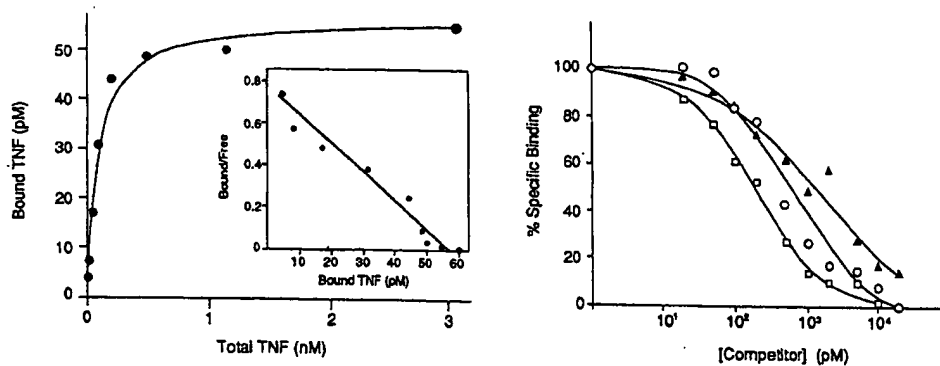


FIG. 2. Binding of TNFR-IgG to TNF- α and TNF- β . (Left) Saturation analysis of TNFR-IgG binding to human TNF- α . Purified TNFR-IgG was immobilized in microtiter wells coated with anti-IgG Fc antibody and incubated with increasing concentrations of recombinant human 125 I-TNF- α . Saturation and Scatchard (Inset) plots were generated by using the best fit as determined by unweighted least-squares regression analyses. (Right) Competition analysis of TNFR-IgG binding to recombinant human TNF- α (○), human TNF- β (▲), and murine TNF- α (□). TNFR-IgG was incubated with 100 pM 125 I-TNF- α in the presence of increasing concentrations of unlabeled competitor.

that both TNFs bind to type 1 TNFR (4–8). A K_d of 550 ± 100 pM was observed for TNF- β and a K_d of 75 ± 5 pM was observed for murine TNF- α (Fig. 2 Right). Notably, the K_d for binding of TNFR-IgG to TNF- α was significantly lower than values reported for type 1 cell-surface or soluble TNFR (sTNFR) (470–660 nM) (4–6). Thus, TNFR-IgG appears to bind to TNF- α with 6- to 8-fold higher affinity than type 1 cell surface TNFR or sTNFR. This higher affinity may be due to a multivalent interaction between TNFR-IgG and TNF- α , as the structure of TNFR-IgG is dimeric (Fig. 1) and the structure of TNF- α is trimeric (22–24). Indeed, saturation analysis in solution, in which complexes of 125 I-TNF- α and TNFR-IgG were precipitated quantitatively with protein A, showed a molar binding ratio of trimeric TNF- α and TNFR-IgG of $1.25 \pm 0.05:1$ (not shown). These results are consistent with the possibility that the two TNFR domains of a TNFR-IgG molecule interact with one TNF- α trimer, which may result in a more stable binding interaction. Alternatively, only one of two TNFR domains in TNFR-IgG may interact with a TNF- α trimer; this is less likely, however, since it would not be expected to result in higher binding affinity.

TNFR-IgG Blocks the Cytolytic Actions of TNF- α and TNF- β *in Vitro*. To test the ability of TNFR-IgG to antagonize TNF activity *in vitro*, we investigated the effect of TNFR-IgG on the induction of cell lysis by TNF- α in actinomycin D-treated murine L-M cells (Fig. 3 Left). While no inhibitory effect was observed with CD4-IgG, TNFR-IgG was able to

block cell killing completely, with 50% inhibition (IC_{50}) occurring at $0.5 \mu\text{g/ml}$ (5 nM). For comparison, we tested type 1 sTNFR and found an IC_{50} of $3.5 \mu\text{g/ml}$ (120 nM). In addition, we tested the activity of two highly neutralizing monoclonal antibodies to human TNF- α (25) and found an IC_{50} of $\approx 3.5 \mu\text{g/ml}$ (≈ 21 nM). Thus, on a molar basis, TNFR-IgG was 24-fold more efficient than sTNFR and 4.2-fold more efficient than anti-TNF- α antibodies in blocking the cytolytic action of TNF- α . We tested also the ability of TNFR-IgG to block the cytolytic activity of TNF- β (Fig. 3 Right). Complete inhibition of cell killing was achievable, with an IC_{50} of $1.5 \mu\text{g/ml}$ (15 nM). Thus, TNFR-IgG was less efficient by a factor of 3 in blocking TNF- β than TNF- α , consistent with its lower affinity for TNF- β . These results show that TNFR-IgG acts as a full antagonist *in vitro* against both types of TNF.

TNFR-IgG Protects Against Septic Shock in Mice. To investigate the ability of TNFR-IgG to act as a TNF antagonist *in vivo*, we used a model for septic shock in mice (Fig. 4). In animals receiving an LD_{100} dose of endotoxin, complete lethality was observed within 48 hr. Injection of TNFR-IgG 0.5 hr prior to endotoxin administration prevented lethality at a TNFR-IgG dose of $20 \mu\text{g}$ per mouse and provided partial protection at lower doses, whereas CD4-IgG had no significant effect (Fig. 4 Left). We investigated the temporal relation of TNFR-IgG and endotoxin injection also (Fig. 4 Right). Injection of $10 \mu\text{g}$ of TNFR-IgG per mouse provided signif-

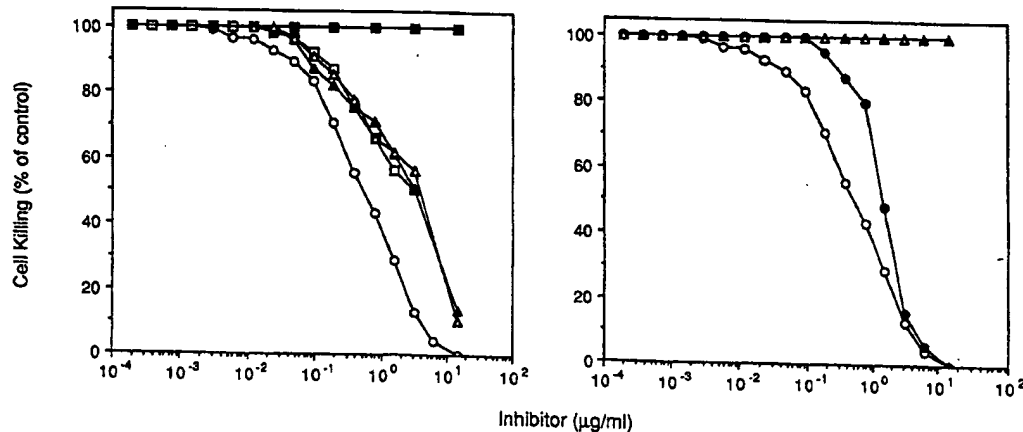


FIG. 3. Inhibition of TNF cytotoxicity by TNFR-IgG *in vitro*. (Left) Effect of TNFR-IgG (○), soluble type 1 TNFR (□), monoclonal antibodies D (Δ) or E (▲) to human TNF- α (15), or CD4-IgG (■) (11) on the killing of actinomycin D-treated murine L-M cells induced by TNF- α (1 ng/ml). (Right) Effect of TNFR-IgG (○, ●) or CD4-IgG (Δ, ▲) on cell killing by TNF- α (1 ng/ml) (○, Δ) or TNF- β (1 ng/ml) (●, ▲).

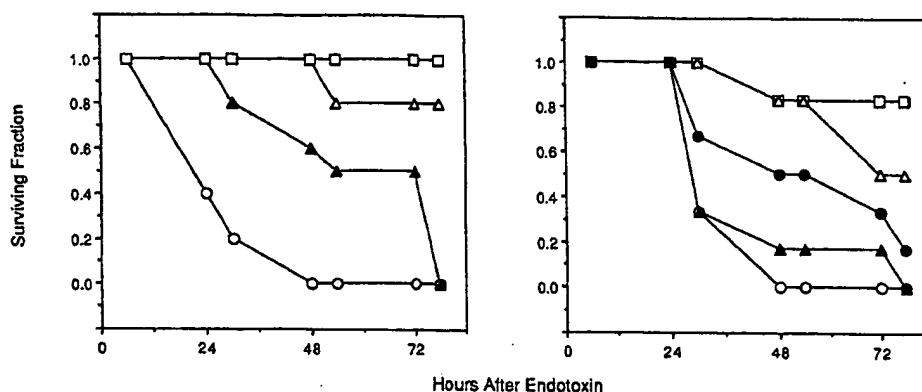


FIG. 4. Inhibition of endotoxin-induced lethality in mice by TNFR-IgG. (Left) Effect of the dose of TNFR-IgG on endotoxin-induced lethality. Mice ($n = 10$) were given an i.v. injection of TNFR-IgG at a dose per mouse of 20 μ g (□), 4 μ g (Δ), and 0.8 μ g (▲) or CD4-IgG at 4 μ g per mouse (○). Thirty minutes later, the mice were given an i.v. injection of endotoxin. Survival data is shown for the first 78 hr and remained unchanged for at least another week. (Right) Effect of the time of administration of TNFR-IgG in relation to the time of endotoxin challenge. Mice ($n = 6$) were given i.v. injection of endotoxin only (○) or of TNFR-IgG (10 μ g per mouse) 0.5 hr before (□), 0.5 hr after (Δ), 1 hr after (●), or 2 hr after (▲) administration of endotoxin.

icant protection 0.5 hr before, 0.5 hr after, or 1 hr after endotoxin injection but little protection 2 hr after endotoxin injection. These data show that TNFR-IgG can prevent or significantly delay endotoxin-induced lethality in mice when given prior to or shortly after endotoxin challenge.

DISCUSSION

Our results show that TNFR-IgG, a molecule that combines the TNF binding function of the extracellular portion of type 1 TNFR with the dimeric structure of IgG, is a potent TNF antagonist. At the molecular level, TNFR-IgG exhibits significantly higher affinity for TNF- α than monomeric cell surface or soluble TNFR, and a molar binding ratio of 1.25:1 TNF- α trimer to TNFR-IgG suggests that this higher affinity may be due to bivalent binding to TNF- α .

At the cellular level, TNFR-IgG blocks the cytolytic action of TNF- α or TNF- β in murine L-M cells completely, and is markedly more potent than sTNFR or anti-TNF- α monoclonal antibodies in blocking TNF- α . The difference in TNF- α binding affinity between TNFR-IgG and sTNFR probably contributes to the differential efficiency of these forms of TNFR in blocking the TNF- α cytolytic activity. However, the difference between TNFR-IgG and sTNFR in blocking TNF- α (24-fold) is significantly greater than the difference in affinity (6- to 8-fold). Previous work with anti-TNFR antibodies showed that bivalent but not monovalent antibody fragments can activate TNFR (26), indicating that a TNF- α trimer may trigger signal transduction by cross-linking two cell surface TNFR molecules. Therefore, the ability of TNFR-IgG to block two receptor binding sites on a TNF- α trimer simultaneously, thus rendering TNF- α unable to dimerize cell surface receptors, also may contribute to the greater efficiency of TNFR-IgG vs. sTNFR in blocking TNF- α .

At the level of the whole organism, TNFR-IgG can prevent or protect against endotoxic shock in mice, depending on the dose and time of injection. This confirms the hypothesis that TNF- α is a key contributor to the septic shock syndrome, first suggested by the ability of anti-TNF- α antibodies to protect against septic shock (9, 10). The ability of TNFR-IgG to provide protection at the doses tested in this study appears limited to about 1 hr after endotoxin challenge. This is consistent with the finding that the rise in circulating levels of TNF- α in animals challenged with endotoxin or *E. coli* and in patients with septic shock is transient (13-17). Taken together, these observations support the notion that the tran-

sient increase in TNF- α following sepsis triggers a subsequent cascade of events that can lead to the pathogenesis of shock and multiple organ failure.

In the past few decades, major advances in the treatment of bacterial infections have been achieved, such as the development of powerful antimicrobial agents. Nonetheless, the number of cases with sepsis and the rate of mortality remain high (27). Recently, a monoclonal antibody to endotoxin, derived from human sources, has been shown to be partially protective in patients with septic shock (28). Another approach to the treatment of sepsis has been the administration of murine anti-TNF- α monoclonal antibodies (29). However, the use of murine antibodies in humans leads to the generation of anti-murine antibodies (29), which could hamper the action of the anti-TNF- α antibodies during repeated or chronic administration.

The observation that TNFR-IgG provides protection against endotoxin-induced lethality when given before and shortly after endotoxin administration suggests that this molecule may offer clinical potential both prophylactically in patients at high risk of sepsis and therapeutically in patients with shock. In contrast to murine anti-TNF- α antibodies, TNFR-IgG is derived from human proteins and therefore is expected to be much less immunogenic in humans, as indeed is the case for the similarly constructed CD4-IgG (A.A. and D.J.C., unpublished results). In addition, the increased affinity of TNFR-IgG for TNF- α appears to confer greater efficiency in blocking TNF- α *in vitro*, as compared with sTNFR or anti-TNF- α antibodies, although this remains to be investigated *in vivo*. Finally, since the rise in circulating TNF- α is subsequent to the occurrence of endotoxemia, it may be possible to extend the time window for treatment of septic shock by combination therapy with anti-endotoxin antibodies and anti-TNF- α agents such as TNFR-IgG.

We thank David Peers for help in TNFR-IgG purification, Greg Bennet for anti-TNFR antibodies, Kathy Kosewicz and Laura Closskey for assay support, Dr. Chris Clark for comments on the manuscript, and Carol Morita for graphics.

- Goeddel, D. V., Aggarwal, B. B., Gray, P. W., Leung, D. W., Nedwin, G. E., Palladino, M. A., Patton, J. S., Pennica, D., Shepard, H. M., Sugarman, B. J. & Wong, G. H. W. (1986) *Cold Spring Harbor Symp. Quant. Biol.* 51, 597-609.
- Beutler, B. & Cerami, A. (1989) *Annu. Rev. Immunol.* 7, 625-655.
- Grumfeld, C. & Palladino, M. A. (1990) *Adv. Intern. Med.* 35, 45-71.

4. Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H. W., Gatanaga, T., Granger, G. A., Lentz, R., Raab, H., Kohr, W. J. & Goeddel, D. V. (1990) *Cell* 61, 361-370.
5. Loetscher, H., Pan, Y. C. E., Lahm, H. W., Gentz, R., Brockhaus, M., Tabuchi, H. & Lesslauer, W. (1990) *Cell* 61, 351-359.
6. Smith, C. A., Davis, T., Anderson, D., Solam, L., Beckman, M. P., Jerzy, R., Dower, S. K., Cosman, D. & Goodwin, R. G. (1990) *Science* 248, 1019-1023.
7. Nophar, Y., Kemper, O., Brakebusch, C., Engelman, H., Zwang, R., Aderka, D., Holtmann, H. & Wallach, D. (1990) *EMBO J.* 9, 3269-3278.
8. Kohno, T., Brewer, M. T., Baker, S. L., Schwartz, P. E., King, M. W., Hale, K. K., Squires, C. H., Thompson, R. C. & Vannice, J. L. (1990) *Proc. Natl. Acad. Sci. USA* 87, 8331-8335.
9. Beutler, B., Milsark, I. W. & Cerami, A. (1985) *Science* 229, 869-871.
10. Tracey, K. J., Fong, Y., Hesse, D. G., Manogue, K. R., Lee, A. T., Kuo, G. C., Lowry, S. F. & Cerami, A. (1987) *Nature (London)* 330, 662-664.
11. Starnes, H. F., Jr., Warren, R. S., Jeevanandam, M., Gabri- love, J. L., Larchian, W., Oettgen, H. F. & Brennan, M. F. (1988) *J. Clin. Invest.* 82, 1321-1325.
12. Michie, H. R., Spriggs, D. R., Manogue, K. R., Sherman, M. L., Revhaug, A., O'Dwyer, S. T., Arthur, K., Dinarello, C. A., Cerami, A., Wolff, S. M., Kufle, D. W. & Wilmore, D. W. (1988) *Surgery* 104, 280-286.
13. Michie, H. R., Manogue, K. R., Spriggs, D. R., Revhaug, A., O'Dwyer, S., Dinarello, C. A., Cerami, A., Wolff, S. M. & Wilmore, D. W. (1988) *N. Engl. J. Med.* 318, 1481-1486.
14. Silva, A. T., Bayston, K. F. & Cohen, J. (1990) *J. Infect. Dis.* 162, 421-427.
15. Waage, A., Halstensen, A. & Espevik, T. (1987) *Lancet* i, 355-357.
16. Girardin, E., Grau, G. E., Dayer, J. M., Roux-Lombard, P. & Lambert, P. H. (1988) *N. Engl. J. Med.* 319, 397-400.
17. Debets, J. M. H., Kampmeijer, R., van der Linden, P. M. H., Buurman, W. A. & van der Linden, C. J. (1989) *Crit. Care Med.* 17, 489-494.
18. Capon, D. J., Chamow, S. M., Mordenti, J., Marsters, S. A., Gregory, T. J., Mitsuya, H., Byrn, R. A., Lucas, C., Wurm, F. M., Groopman, J. R. & Smith, D. H. (1989) *Nature (London)* 337, 525-531.
19. Byrn, R. A., Mordenti, J., Lucas, C., Smith, D. H., Marsters, S. A., Johnson, J. S., Cossum, P., Chamow, S. M., Wurm, F. M., Gregory, T. J., Groopman, J. E. & Capon, D. J. (1990) *Nature (London)* 344, 667-670.
20. Ashkenazi, A., Presta, L. G., Marsters, S. A., Camerato, T. R., Rosenthal, K. R., Fendly, B. M. & Capon, D. J. (1990) *Proc. Natl. Acad. Sci. USA* 87, 7150-7154.
21. Kawade, Y. & Watanabe, Y. (1984) *J. Interferon Res.* 4, 571-584.
22. Jones, E. Y., Stuart, D. I. & Walker, N. P. C. (1989) *Nature (London)* 338, 225-228.
23. Eck, M. J. & Sprang, S. R. (1989) *J. Biol. Chem.* 264, 17595-17605.
24. Van Ostade, X., Tavernier, J., Prange, T. & Fiers, W. (1991) *EMBO J.* 10, 827-836.
25. Bringman, T. S. & Aggarwal, B. B. (1987) *Hybridoma* 6, 489-507.
26. Engelman, H., Holtman, H., Brakebusch, C., Shemer Avni, Y., Sarov, I., Nophar, Y., Hadas, E., Leitner, O. & Wallach, D. (1990) *J. Biol. Chem.* 265, 14497-14504.
27. Wolff, S. M. (1991) *N. Engl. J. Med.* 324, 486-488.
28. Ziegler, E. J., Fisher, C. J., Sprung, C. L., Straube, R. C., Sadoff, J. C., Foulke, G. E., Wortel, C. H., Fink, M. P., Dellinger, R. P., Teng, N. N. H., Allen, I. E., Berger, H. J., Knatterud, G. L., LoBuglio, A. F. & Smith, C. R. (1991) *N. Engl. J. Med.* 324, 429-436.
29. Exley, A. R., Cohen, J., Buurman, W., Owen, R., Lumley, J., Hanson, G., Aulakh, J. M., Bodmer, M., Stephens, S., Riddell, A. & Perry, M. (1990) *Lancet* 335, 1275-1277.

8. Inoue, Y., Nakamura, R., Mikoshiba, K. & Tsukada, Y. *Brain Res.* 219, 85-94 (1981).
9. Sharkey, R. G., Benesi, D. & Catterall, W. A. *Biochemistry* 23, 6078-6086 (1984).
10. Schmidt, J., Rossie, S. & Catterall, W. A. *Proc. natn. Acad. Sci. U.S.A.* 82, 4847-4851 (1985).
11. Stumer, W., Methfessel, C., Sakman, B., Noda, M. & Numa, S. *Eur. Biophys. J.* 14, 131-138 (1987).
12. Sontheimer, H., Trotter, J., Schachner, M. & Kettenman, H. *Neuron* 2, 1135-1145 (1989).
13. Barres, B. A., Chun, L. L. Y. & Corey, D. P. *Neuron* 2, 1375-1388 (1989).
14. Sheedio, H. J. & Siegel, G. J. *Brain Res.* 415, 105-114 (1987).
15. Waxman, S. G., Black, J. A., Duncan, I. D. & Ransom, B. R. *J. Neurocytol.* 19, 11-27 (1990).
16. Waxman, S. G., Black, J. A., Kocsis, J. D. & Ritchie, J. M. *Proc. natn. Acad. Sci. U.S.A.* 86, 1406-1410 (1989).
17. Scheirman, R. I. et al. *J. Biol. Chem.* 264, 1060-1066 (1989).
18. Chiu, S. Y. & Schwarz, W. J. *Physiol.* 391, 631-649 (1987).
19. Caroni, P. & Schwab, M. E. *J. Cell. Biol.* 106, 1281-1288 (1988).
20. Hodgkin, A. L. *Phil. Trans. R. Soc. Lond. B* 270, 297-300 (1975).
21. Campagnoni, A. T. *J. Neurochem.* 51, 1-14 (1988).
22. Lemke, G. *Neuron* 1, 535-543 (1988).
23. Sorg, B. J., Smith, M. M. & Campagnoni, T. J. *Neurochem.* 49, 1146-1154 (1987).
24. Frail, D. E. & Braun, P. E. *J. Neurochem.* 45, 1071-1075 (1985).
25. Wisniewski, H. M., Oppenheimer, D. & McDonald, W. E. *J. Neuropathol. exp. Neurol.* 35, 327 (1976).
26. Smith, K. J. & McDonald, W. E. *Nature* 286, 154-155 (1980).
27. Offard, J. & Catterall, W. A. *Neuron* 2, 1447-1452 (1989).
28. Lidow, M. S., Goldman-Rakic, P. S., Rakic, P. & Gallagher, D. W. *Brain Res.* 459, 105-119 (1988).
29. Bird, T. D., Farrell, D. F. & Sumo, S. M. *J. Neurochem.* 31, 387-391 (1978).
30. Mourre, C., Moll, C., Loubet, A. & Lazdunski, M. *Brain Res.* 448, 128-139 (1988).

ACKNOWLEDGEMENTS. We thank H. D. Shine for electron microscopy of shiverer optic nerve. This work was supported by March of Dimes/Birth Defects Foundation, NICHD, Blue Bird Circle Foundation for Pediatric Neurology, and a Pew Biomedical Scholars Award (U.L.N.).

Prevention of HIV-1 IIIB infection in chimpanzees by CD4 immunoadhesin

Rebecca H. R. Ward, Daniel J. Capon*, Catherine M. Jett†, Krishna K. Murthy†, Joyce Mordenti, Catherine Lucas, Steve W. Frie, Alfred M. Prince‡, James D. Green & Jorg W. Eichberg†§

Genentech Inc., 460 Pt San Bruno Boulevard, S. San Francisco, California 94080, USA

* Cell Genesys Inc., 344 Lakeside Drive, Foster City, California 94404, USA

† Southwest Foundation for Biomedical Research, San Antonio, Texas 78284, USA

‡ The Lindsley F. Kimball Research Institute of the NY Blood Center, 310 East 67th Street, New York 10021, USA

§ Present address: Wyeth-Ayerst Research, 145 King of Prussia Road, Philadelphia, Pennsylvania 19087, USA

THE first step in infection by the human immunodeficiency virus (HIV) is the specific binding of gp120, the envelope glycoprotein of HIV, to its cellular receptor, CD4 (see ref. 1 for review). To inhibit this interaction, soluble CD4 analogues that compete for gp120 binding and block HIV infection *in vitro* have been developed²⁻⁸. To determine whether these analogues can protect an uninfected individual from challenge with HIV, we used the chimpanzee model system of cell-free HIV infection. Chimpanzees are readily infected with the IIIB strain of HIV-1, becoming viraemic within about 4-6 weeks of challenge, although they do not develop the profound CD4⁺ T-cell depletion and immunodeficiency characteristic of HIV infection in humans⁹. CD4 immunoadhesin (CD4-IgG), a chimaeric molecule consisting of the N-terminal two immunoglobulin-like regions of CD4 joined to the Fc region of human IgG1 (refs 8, 10), was selected as the CD4 analogue for testing because it has a longer half-life than CD4, contributed by the IgG Fc portion of the molecule. In humans, this difference results in a 25-fold increased concentration of CD4-IgG in the blood compared with recombinant CD4 (ref. 11). Here we report that pretreatment with CD4-IgG can prevent the infection of chimpanzees with HIV-1. The need for a preventative agent is particularly acute in perinatal HIV transmission. As recombinant CD4-IgG, like the parent IgG molecule, efficiently

immune state in a fetus before HIV transfer occurs, thus preventing infection.

Two chimpanzees were pretreated with CD4-IgG (5 mg kg⁻¹ given intravenously (i.v.)) at 8 h and 1 h before challenge, then inoculated with 120 tissue culture infectious doses (TCID₅₀s) of HIV-1 IIIB (30 chimp infectious doses). After challenge, the animals received further CD4-IgG treatment for 9 weeks. A control animal was similarly challenged but not treated. This dose of HIV-1 IIIB is expected to result in seroconversion of all untreated animals within 9 weeks (see, for example, ref. 12). The serum concentrations of CD4-IgG were predicted from the known human pharmacokinetics and checked by enzyme-linked immunosorbent assay (ELISA) where indicated (Fig. 1). The serum concentrations of CD4-IgG attained during the first 2 days of treatment were estimated to be 50-200 µg ml⁻¹, and over the next 5 weeks were predicted to be 5-120 µg ml⁻¹; trough concentrations were confirmed by ELISA. When doses were given i.v. (week 6 onwards), the predicted concentrations of 0.5-200 µg ml⁻¹ were confirmed by ELISA. Treated animals were also monitored for the development of antibodies to CD4-IgG by indirect ELISA. Chimpanzee 37 transiently showed just detectable antibody levels at weeks 7-13 (not shown). Chimpanzee 43 had no detectable antibody response.

Chimpanzees were monitored for HIV infection by detection of virus and by analysis for seroconversion. Virus was detected by a viral coculture assay, in which p24 production was measured, and by the polymerase chain reaction (PCR). The development of antibodies to HIV proteins was assessed by western blot and ELISA whole-virus based assays. The control animal became infected 3 weeks after challenge, as shown by viral coculture (Table 1), and virus could be detected by PCR at 11 weeks. Antibody to the viral protein p24 became evident in this animal by western blot at week 7 (Fig. 2), and subsequently antibodies to other viral proteins, including gp120, were detected. The animal showed seroconversion to HIV by ELISA at week 7 (Table 1). By contrast, the CD4-IgG-treated animals have not shown any signs of infection, after 47 weeks. A single sample from animal 37 (week 23) was positive in two of three PCR assays using a gag primer pair (Table 1), but negative using a pair of *env*-derived primers of identical sensitivity (Table 1 legend). Further samples from the same animal up to week 47 have repeatedly assayed negative by both PCR and viral culture. It thus seems that both treated animals were protected from infection.

Immunization with recombinant gp120 (ref. 13) or successive immunization with a variety of immunogens including the V3 loop of gp120 (ref. 14) can induce protective immunity in chimpanzees against later challenge with HIV-1 IIIB. But passive protection from HIV infection has not been previously shown. Protection of chimpanzees against HIV-1 by pretreatment with a single high dose of purified hyperimmune gamma-globulin (HIVIG) obtained from AIDS patients has been observed (A.M.P. et al., manuscript in preparation). The control animal described here was shared with the HIVIG study. In our study, treatment with CD4-IgG continued for 9 weeks, in contrast to the single dose of HIVIG. Further work is needed to determine the necessary dose and length of treatment with CD4-IgG.

Azidothymidine (AZT), the only drug currently approved for the treatment of AIDS, has not yet been shown to prevent infection by HIV or simian immunodeficiency virus (SIV) in animal models, whether given before¹⁵ or after¹⁶ virus challenge, although it does delay the spread of infection. It is unclear whether AZT can prevent infection after accidental challenge with HIV or maternal-fetal transfer in humans; AZT failures in both forms of challenge are known^{17,18}. Clinical trials to address these issues are under way.

Although our results, and those of others (A.M.P. et al., manuscript in preparation) show that it is possible to prevent

TABLE 1 Detection of HIV infection

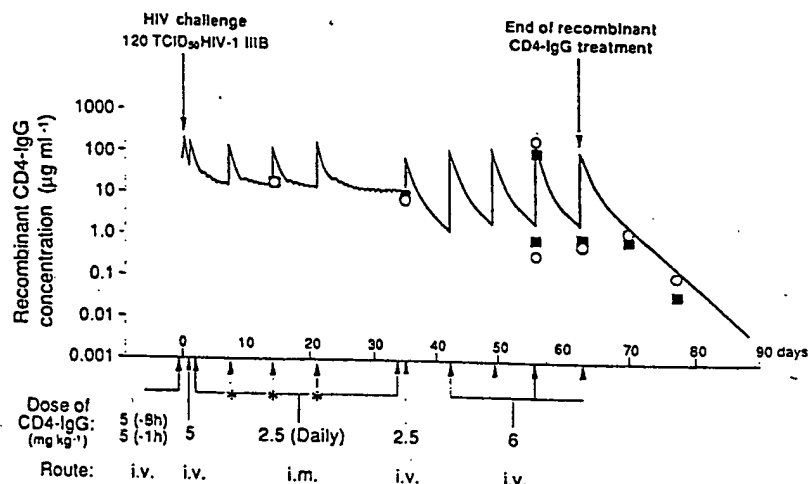
Week	ELISA	Animal 62 (control) p24	PCR	ELISA	Animal 37 (CD4-IgG) p24	PCR	ELISA	Animal 43 (CD4-IgG) p24	PCR
0	<400	—	ND	<400	—	ND	<400	—	ND
1	<400	—	—	<400	—	—	<400	—	—
2	<400	—	—	<400	—	—	<400	—	—
3	<400	+	—	<400	—	—	<400	—	—
5	<400	+	ND	<400	—	ND	<400	—	—
7	400	+	ND	<400	—	—	<400	—	ND
9	6400	+	ND	<400	—	ND	<400	—	—
11	3200	+	+	<400	—	—	<400	—	ND
13	>6400	+	+	<400	—	—	<400	—	—
15	6400	+	+	<400	—	—	<400	—	—
19	12800	+	+	<400	—	—	<400	—	—
23	12800	+	+	<400	—	+	<400	—	—
27	12800	+	+	<400	—	—	<400	—	—
31	12800	+	+	<400	—	—	<400	—	—
35	>6400	+	+	<400	—	—	<400	—	—
39	12800	+	+	<400	—	—	<400	—	—
43	>6400	+	+	<400	—	—	<400	—	—
47	>6400	+	+	<400	—	—	<400	—	—

Detection of HIV infection by antibody ELISA and viral culture. Antibody titres against HIV-1 (first column) were determined using a commercial liquid-phase ELISA (Genetics Systems). Cocultivation of HIV was done as described²² for 4 weeks and infection was detected by assaying for viral p24 antigen (second column). For PCR analysis (third column), DNA was extracted from peripheral blood mononuclear cells using digestion buffer (10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, 1% Tween-20, 1% Nonidet P-40 and 120 µg ml⁻¹ proteinase K). Lysate was incubated at 60 °C for 1 h and extracted with phenol and chloroform. The resulting DNA was solubilized in 25 mM Tris-HCl, pH 7.5. PCR was done essentially as described by Saiki *et al.*²³ for 35 cycles, using the SK 38/39 primer pair, which is specific for the nucleotide sequence of the p24 core protein. Ten copies of proviral DNA can be detected in this assay, based on titration using the Perkin-Elmer HIV-1 PCR kit. Liquid hybridization was done to detect positive samples. Amplified sample (10 µl) was incubated with 1.25 ng ³²P-labelled SK19 probe (specific activity 10⁷ µg⁻¹) and 1.25 µl ReACT buffer at 95 °C for 10 min and snap-cooled. The tubes were incubated at 56 °C for 30 min and centrifuged briefly, loading buffer was added and the samples were applied to a 12% polyacrylamide gel. Positive samples were detected by autoradiography. The sample from animal 37 (week 23) was also subjected to PCR analysis using primer pairs SK68/69, detected by hybridization with probe SK70, with negative results. DNA (1–3 µg) in 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.032 mM each dNTP, 50 ng each primer and 1 unit Taq polymerase was amplified 40 times in a cycle consisting of 94 °C, 1 min; 56 °C, 1 min; 72 °C, 1.5 min. The sensitivity of this PCR reaction is identical to that of the SK38/39 pair described above (10 copies).
ND, not determined.

whether CD4-IgG itself will be clinically effective in this mode. It will be important to test the ability of CD4-IgG to protect against HIV-1 infection using different strains of HIV-1, as some primary patient isolates of HIV-1 are considerably less sensitive than HIV-1 IIIB to inhibition by CD4 derivatives¹⁹. Also, the challenge was given as cell-free virus; cell-associated virus may

be more difficult to inhibit. Nevertheless, these results offer hope for the use of CD4-IgG in the prevention of HIV infection in unborn infants. Currently 15–45% of the infants born to HIV-infected women are themselves infected (see, for example, ref. 22), and no drug is known to decrease transmission. Although there is as yet no definitive information on the time at which

FIG. 1 Predicted blood concentrations of CD4-IgG over the course of treatment. CD4-IgG (5 mg per kg bodyweight) was given i.v. for the first three doses (–8, –1 and +24 h postchallenge). For the following 5 weeks, daily injections of 2.5 mg kg⁻¹ CD4-IgG were given intramuscularly (i.m.). On days when the animals were anaesthetized for blood sampling (days 7, 14 and 21), a 5 mg kg⁻¹ dose was given i.v. (*); on day 35 a 2.5 mg kg⁻¹ dose was given i.v. In weeks 6–9, an i.v. dose of 6 mg kg⁻¹ was given once per week to reduce the stress on the animals. Weights for chimpanzees 37 and 43 were 63 and 43 kg, respectively. The concentration of CD4-IgG was estimated by assuming that chimpanzee pharmacokinetics closely mimic those observed in humans (T. Hodges, J. D. Allan, J. Kahn and J. Groopman, personal communication), as the body weights are close. For i.v. administration, the CD4-IgG serum concentrations (C) were predicted by dose-adjusting the pharmacokinetics observed in humans following 1 mg kg⁻¹ i.v. bolus injection, which are given by $C(\mu\text{g ml}^{-1}) = 17.5 e^{(-0.059t)} + 3.1 e^{(-0.014t)}$, where time (t) is in hours, using a multiple dose simulation routine²¹. For i.m. administration, a 9-h adsorption half-life and 25% bioavailability were assumed. Squares and circles show serum concentrations of CD4-IgG in chimpanzees 43 and 37, respectively, as measured in a double-sandwich ELISA using two monoclonal antibodies to the CD4 portion of the molecule. The first antibody was passively adsorbed into 96-well microtitre plates in NaHCO₃ buffer (0.05M), pH 9.6, overnight at 4 °C. After washing 3 times with PBS containing 0.05% Tween-20 (wash buffer), the antibody was blocked with PBS containing 0.5% BSA and 0.05%



20 for 1 h at room temperature and washed again. Standards, controls and samples diluted in primate serum diluent were added for 2 h at room temperature. After washing 3 times in wash buffer, the second antibody, conjugated to horseradish peroxidase, was added for 1 h at room temperature. Orthophenylenediamine was used as a substrate and plates were

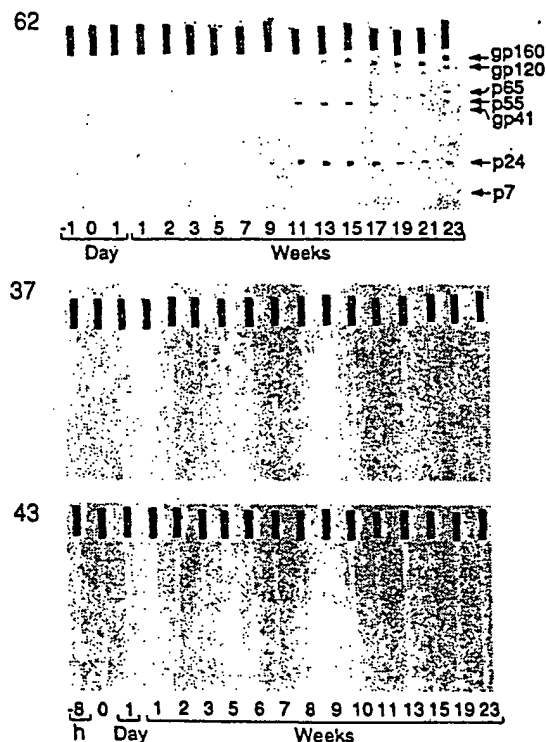


FIG. 2 Western blot seroanalysis of treated (numbers 37 and 43) and control (no. 62) chimps. Samples of serum taken at different times during the protocol were diluted and incubated with commercial (Bio-Rad) western blot strips. The strips were incubated with alkaline phosphate-coupled anti-human IgG (Cappel) and developed using a phosphatase substrate system (Kirkegaard and Perry Laboratories) under conditions recommended by the manufacturers. Samples up to week 47 post-challenge gave identical results (not shown).

fetal infection occurs, an analogy with hepatitis B infection would suggest that most infection occurs late in the third trimester of pregnancy or around the time of delivery. CD4-IgG, like the parent IgG molecule, is transported across the placental barrier during pregnancy in a rhesus monkey model⁹. Thus it may be possible to provide a protective level of CD4-IgG in the fetus before infection with HIV occurs. □

Received 27 February; accepted 10 June 1991.

- Capon, D. & Ward, R. H. R. *A. Rev. Immun.* **9**, 649-678 (1991).
- Smith, D. H. et al. *Science* **228**, 1704-1707 (1987).
- Fisher, R. et al. *Nature* **331**, 76-78 (1988).
- Hussey, R. et al. *Nature* **331**, 78-81 (1988).
- Deen, K. et al. *Nature* **331**, 82-84 (1988).
- Trautwein, A., Luke, W. & Karjalainen, K. *Nature* **331**, 84-86 (1988).
- Berger, E., Fuerst, T. & Moss, B. *Proc. natn. Acad. Sci. U.S.A.* **85**, 2357-2361 (1988).
- Capon, D. et al. *Nature* **337**, 525-531 (1989).
- Eichberg, J. W. in *Animal Models in AIDS* (eds Schellekens, H. & Horzinek, M. C.) 47-52 (Elsevier, Amsterdam, 1990).
- Byrn, R. et al. *Nature* **344**, 667-670 (1990).
- Mordenti, J., Chen, S. A., Moore, J. A., Ferraiolo, B. & Green, J. D. *Pharmac. Res.* (in the press).
- Hu, S.-L. et al. *Nature* **328**, 721-723 (1987).
- Berman, P. W. et al. *Nature* **345**, 622-625 (1990).
- Girard, M. et al. *Proc. natn. Acad. Sci. U.S.A.* **88**, 542-546 (1991).
- McGuire, J. M., Namikawa, R., Shih, C. C., Rabin, L. & Kaneshima, H. *Science* **247**, 564-566 (1990).
- Marrin, L. N., Murphy-Corb, M., Soike, K. F. & Davidson-Fairburn, B. *8th A. Symp. Nonhuman Primate Models for AIDS* 56 (Delta Regional Primate Research Center, Tulane University, 1990).
- Lange, J. M. A. et al. *New Engl. J. Med.* **322**, 1375-1377 (1990).
- Bernard, N. et al. *New Engl. J. Med.* **323**, 916 (1990).
- Daar, E. S., Li, X. L., Moudgil, T. & Ho, D. D. *Proc. natn. Acad. Sci. U.S.A.* **87**, 6574-6578 (1990).
- Blanche, S. et al. *New Engl. J. Med.* **320**, 1643-1648 (1989).
- Thompson, G. & Shumaker, R. *Drug Metabolism Rev.* **21**, 463-469 (1989).
- Anderson, K. P. et al. *J. Infect. Dis.* **160**, 960-969 (1989).
- Soike, R. K. et al. *Science* **230**, 487-491 (1985).

ACKNOWLEDGEMENTS. We thank P. Berman, T. Gregory and A. Ammann for helpful discussions, the Process Sciences Department of Genentech Inc., for providing purified material, Michelle Sanda and the Assay Services Department, Genentech Inc., for assay support, E. K. Cobb, S. R. Rouse and G. I. Melendez for technical assistance and Carol Morita for assistance with figures. This study was supported in part by the NHLBI.

Prevention of HIV-2 and SIV_{sm} infection by passive immunization in cynomolgus monkeys

Per Putkonen, Rigmor Thorstensson, Lili Ghavamzadeh, Jan Albert*, Kerstin Hild, Gunnel Biberfeld & Erling Norrby†

Department of Immunology and * Department of Virology, National Bacteriological Laboratory, S-10521 Stockholm, Sweden
† Department of Virology, Karolinska Institute, S-10521 Stockholm, Sweden

INFECTION of macaques with simian immunodeficiency virus (SIV)^{1,2} and human immunodeficiency virus type 2 (HIV-2)^{3,4} are useful models for studies of immunotherapy and vaccination against HIV as well as for testing of antiviral drugs. Vaccine research showing protective immunity in immunized monkeys⁴⁻¹⁰ has indicated that it will be possible to develop a vaccine for prevention of human HIV infection, although many hurdles remain. The design of an HIV vaccine would be helped if the basis of the protective immunity could be elucidated. Passive immune prophylaxis offers a means to determine the relative role of antibodies in protection against infection. We have studied whether a transfer of antibodies can prevent HIV-2 and SIV_{sm} (SIV of sooty mangabey origin) infection in cynomolgus monkeys. Sera with high antibody titres were collected, heat-treated and injected into naive animals 6 h before challenge with 10-100 monkey-infectious doses of live homologous virus. All control animals treated with normal monkey serum ($n = 6$) or no serum ($n = 39$) became infected by the challenge virus, whereas five out of seven animals pretreated with antibody-containing serum at a dose of 9 ml kg⁻¹ resisted infection. Thus passively transferred antibodies can protect against a low-dose lentivirus challenge in a nonhuman primate.

Two anti-HIV-2 serum pools were obtained from a cynomolgus monkey (A5) (*Macaca fascicularis*) which earlier had been immunized with an inactivated whole-virus vaccine and protected against a homologous HIV-2 challenge⁴. This monkey (A5) received five intramuscular 100 µg doses of whole, inactivated HIV-2_{SBL-6669} emulsified in incomplete Freund's adjuvant on

TABLE 1 Antibody titres in serum pools used for passive immunization of cynomolgus monkeys

Sample	Titres of antibodies in:		
	Whole antigen ELISA	V3 peptide ELISA	Neutralization assay
Anti-HIV-2 serum pool 1	70,000	1,100	1,280
Anti-HIV-2 serum pool 2	270,000	4,800	2,560
Anti-SIV _{sm} serum pool	58,000	10,000	80

The serum pools had antibody reactivity to *gag-pol*- and *env*-encoded structural proteins as detected by immunoblotting using antigen preparation of HIV-2_{SBL-6669} (data not shown). The antibody content of serum pools and passively immunized animals was determined in whole HIV-2_{SBL-6669} viral lysate ELISA¹⁵ and in homologous V3 peptide ELISA¹⁶. The V3 peptide assay was included for antibody determination because a recent study¹⁷ has demonstrated immunogenic dominating linear sites of HIV-2_{SBL-6669} with capacity to induce neutralizing antibodies and cytotoxic antibodies in this region. The neutralizing antibodies were determined by incubation of twofold dilutions of serum and a stock virus preparation of HIV-2_{SBL-6669} or SIV_{sm} before addition of HUT-78 cells. The supernatants were analysed in an HIV-2/SIV antigen assay¹⁸ after 7 days in culture. The neutralizing titre was defined as the reciprocal of the highest dilution giving a 50% reduction in absorbance value in the antigen assay. Sera collected from the monkeys before passive immunization were used as negative controls in the serological assays.

Mechanisms of Receptor-Mediated Rhinovirus Neutralization Defined by Two Soluble Forms of ICAM-1

JEFFREY M. GREVE,* CARLA P. FORTE, CHRISTOPHER W. MARLOR, ANN M. MEYER, HELANA HOOVER-LITTY, DAVID WUNDERLICH, AND ALAN MCCLELLAND

Molecular Therapeutics, Inc., Miles Research Center, 400 Morgan Lane, West Haven, Connecticut 06516

Received 23 May 1991/Accepted 15 August 1991

The majority of human rhinoviruses use intercellular adhesion molecule 1 (ICAM-1) as a cell surface receptor. Two soluble forms of ICAM-1, one corresponding to the entire extracellular portion [tICAM(453)] and one corresponding to the two N-terminal immunoglobulin-like domains [tICAM(185)], have been produced, and their effects on virus-receptor binding, virus infectivity, and virus integrity have been examined. Results from competitive binding experiments indicate that the virus binding site is largely contained within the two N-terminal domains of ICAM-1. Virus infectivity studies indicate that tICAM(185) prevents infection by direct competition for receptor binding sites on virus, while tICAM(453) prevents infection at concentrations 10-fold lower than that needed to inhibit binding and apparently acts at the entry or uncoating steps. Neutralization by both forms of soluble ICAM-1 requires continual presence of ICAM-1 during the infection and is largely reversible. Both forms of soluble ICAM-1 can alter rhinovirus to yield subviral noninfectious particles lacking the viral subunit VP4 and the RNA genome, thus mimicking virus uncoating *in vivo*, although this irreversible modification of rhinovirus is not the major mechanism of virus neutralization.

The majority of human rhinoviruses, the major causative agent of the common cold, utilize intercellular adhesion molecule 1 (ICAM-1) as a receptor on host cells (11). ICAM-1 is an integral membrane protein with a large N-terminal extracellular portion, a transmembrane anchor, and a short C-terminal cytoplasmic domain. The normal physiological function of ICAM-1 is to serve as a membrane-bound ligand of the leukocyte integrin lymphocyte function-associated antigen 1 (LFA-1) and mediate intercellular adhesion between leukocytes and a variety of cell types (17, 18, 33). The protein has sequence homology with members of the immunoglobulin supergene family, and its extracellular domain can be divided into five immunoglobulin-like domains (32, 35). Electron microscopy (34) has indicated that ICAM-1 is a highly elongated molecule.

The 3-dimensional structure of two rhinovirus serotypes have now been determined to atomic resolution by Rossmann and colleagues (15, 25). The virion is composed of a protein capsid of 60 protomeric units, consisting of the four protein subunits (VP1 to VP4), surrounding an RNA genome. Each of the 60 protomeric units possesses a recessed canyon that is likely to contain the receptor binding site (for a review, see reference 24). The dimensions of the canyon are such that it is too small to admit the combining site of an antibody but is apparently large enough to admit the virus binding site of ICAM-1. The precise extent of the virus binding site on ICAM-1 remains to be determined, although results from mouse-human chimeras and site-directed mutagenesis indicate that the N-terminal domain plays a major role in virus binding (20, 34), and a molecular model has been developed for the interaction of the N-terminal domain of ICAM-1 with the rhinovirus canyon (9). Detergent-solubilized transmembrane ICAM-1 binds to rhinovirus in solution (11), and a truncated form of ICAM-1 consisting of the extracellular domain binds to and neutralizes rhinovirus (19).

In an effort to further understand the molecular basis of

rhinovirus-ICAM-1 interaction and to determine the mechanism by which soluble ICAM-1 neutralizes rhinovirus, we have produced two truncated soluble forms of ICAM-1. In this report, the properties of these proteins are described and their abilities to inhibit rhinovirus-receptor binding and infectivity are compared. These data indicate that there are three distinct mechanisms by which soluble ICAM-1 prevents virus infection and have implications for the role of the receptor in virus uncoating within host cells.

MATERIALS AND METHODS

cDNA constructions. Modified forms of the ICAM-1 cDNA were created by polymerase chain reaction (29) by using the full-length ICAM-1 cDNA pHRR-2 (11) as template. Plasmid DNA was digested with *Eco*R1 to excise the ICAM-1 insert and treated with alkaline phosphatase to prevent recircularization of the vector in subsequent ligation steps. Template DNA (10 ng) was subjected to 10 cycles of polymerase chain reaction amplification with the 5' oligonucleotide primer GGAATTCAGCTTCTCAGCCTCGCTATGGCTCCCAG CAGCCCCGGGCC and the following 3' oligonucleotide primers: GGAATTCCTGCAGTCACTCATAACCGGGGGG AGAGCACATT for tICAM(453), TTCTAGAGGATCCTC AAAAGCTGTAGATGGTCACTGTCTG for tICAM(284), TTCTAGAGGATCCTCAAAGGCTCTGGAGCTGGTAGG GGG for tICAM(185), and TTCTAGAGGATCCTCACCCT TCTGGAGTCCAGTACACGG for tICAM(88). The polymerase chain reaction products were digested with either *Eco*R1 [tICAM(453)] or *Eco*R1 and *Bam*H1 [tICAM(284), tICAM(185), and tICAM(88)] and cloned into the polylinker site of Bluescript SK+ (Stratagene). Clones containing the desired inserts were verified by restriction analysis and DNA sequencing. The inserts were excised by digestion with *Hind*III and *Xba*I and inserted into the expression vector CDM8 (30).

Transfections and analysis of secreted proteins. For transient expression, COS cells were transfected by the DEAE-dextran method (16) and labeled 72 h after transfection with

* Corresponding author.

[^{35}S]cysteine in cysteine-free Dulbecco's modified essential medium (DMEM)-2% fetal calf serum for 18 h; culture supernatants were then immunoprecipitated with the anti-ICAM-1 monoclonal antibody c78.4 immunoglobulin G (IgG)-Sephacrose and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (11). Stable CHO transfectants were obtained by cotransfection of ICAM-1 cDNAs with pSV2-dihydrofolate reductase into dihydrofolate reductase-deficient CHO cells by the calcium phosphate method or by electroporation (3). Transfected cells were cloned, and individual clones secreting ICAM-1 protein were identified by radioimmunoassay (RIA) of culture supernatants. Cell lines secreting tICAM(453) (CT.2A) and tICAM(185) (CD12.1A) were selected for further study and were subjected to gene amplification in methotrexate-containing media (3). A clone derived from CT.2A (resistant to 100 nM methotrexate) and a clone derived from CD12.1A (resistant to 1 μM methotrexate) were used for purification of soluble truncated proteins.

RIA. Two monoclonal antibodies, c92.5 (which recognizes the same epitope as c78.4) and c78.5, define two distinct conformational epitopes on ICAM-1 (20). These two antibodies were utilized in an RIA for soluble ICAM-1. c92.5 IgG was absorbed onto Immulon-1 (Dynatech, Inc.) microtiter plates, the plates were blocked by treatment with a solution of 10 mg of bovine serum albumin (BSA) per ml-N buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid]-200 mM NaCl-1 mM CaCl_2 -1 mM MgCl_2 [pH 7.5]), and the plates were incubated with ICAM-1-containing samples. The plates were then washed with N buffer-0.05% Tween 20 and incubated with ^{125}I -c78.5 IgG (labeled with ^{125}I -Bolton-Hunter reagent), and the bound radioactivity was determined after the plates were washed and the radioactivity was solubilized with 1% SDS. The concentration of truncated ICAM-1 was determined by comparison with a standard curve of purified ICAM-1.

Protein purification. ICAM-1 was prepared from detergent lysates of HE1 cells, and tICAM(453) and tICAM(185) were purified from culture supernatants of their respective CHO transfectant cell lines by monoclonal antibody affinity chromatography as described previously (11) and then by either ion-exchange chromatography on Mono-Q for tICAM(453) with absorption in 10 mM Tris (pH 6.0) and elution with a 0 to 0.5 M NaCl gradient or gel filtration on Superose-12 columns (Pharmacia) for tICAM(185). Protein concentration was quantitated by amino acid analysis and by RIA. Amino acid analysis was performed on an Applied Biosystems model 420A amino acid analyzer.

Hydrodynamic properties. The f_{exp}/f_0 values for truncated ICAM-1 proteins were determined from the apparent Stokes' radii (R_s) determined by gel filtration on a Superose-12 column calibrated with protein standards (ferritin, 61.0 nm; catalase, 52.2 nm; bovine serum albumin, 35.5 nm; ovalbumin, 30.5 nm; and RNase A, 16.4 nm) and the calculated molecular weights of the core-glycosylated form of the proteins (determined by SDS-PAGE synthesized in the presence of swainsonin), as follows: $f_{\text{exp}} = 6\pi\eta R_s$ and $f_0 = (v_2 + \delta v_1^0/v_2)^{1/3} f_{\text{min}}$ (frictional coefficient of solvated sphere), where $f_{\text{min}} = 6\pi\eta(3Mv_2/4\pi N_0)^{1/3}$ (frictional coefficient of an unsolvated sphere). The following values are assumed: $v_2 = 0.73 \text{ cm}^3/\text{g}$ (partial specific volume of protein), $v_1^0 = 1.0 \text{ cm}^3/\text{g}$ (partial specific volume of solvent), $\delta = 0.35 \text{ g of H}_2\text{O per g of protein}$ (solvation of protein), $\eta = 0.01 \text{ g/(cm} \cdot \text{s)}$ (viscosity of solvent), and $v_1^0 = 1.0 \text{ cm}^3/\text{g}$ (specific volume of the solvent). The molecular weights of tICAM

(453) and tICAM(185) were assumed to be 64,100 and 27,200, respectively.

CD. Circular dichroism (CD) spectra were recorded on an AVIV model 62DS spectrometer. Protein solutions at approximately 0.5 mg/ml in the indicated buffers were scanned at 20°C in a cell with a 0.1-cm path length. Five repetitive scans (1-nm interval, 1.5-nm bandwidth) were averaged, buffer-subtracted, and then smoothed for each spectrum. Molar ellipticity was calculated by using protein concentrations determined by amino acid analysis. The spectra for c92.5 IgG, tICAM(453), and tICAM(185) were collected in 20 mM sodium phosphate buffer (pH 7.5), and the spectrum for ICAM-1 was collected in 0.1% octylglucoside-150 mM NaCl-10 mM sodium phosphate (pH 7.5).

Virus binding assay. ICAM-1 (100 ng), purified as described previously (11) in the presence of 0.1% beta-octylglucoside, was absorbed to Immulon-4 (Dynatech) microtiter plates by 10-fold dilution into N buffer, and the plates were incubated overnight at 4°C. The ICAM-1-coated plates were washed, blocked with a solution of 10 mg of BSA-N buffer per ml, and then washed extensively with 0.1% Triton X-100-N buffer-1 mg BSA per ml. The absorbed ICAM-1 was stably bound under these conditions and supported the binding of ^{35}S -HRV3. ^{35}S -HRV3 ($2 \times 10^5 \text{ cpm/ml}$) was preincubated with various amounts of ICAM-1 proteins for 30 min at 34°C. A 0.1-ml portion of these samples was added to the wells of the ICAM-1-coated microtiter dish and then incubated for 3 h at 34°C. The plates were washed extensively, and the bound radioactivity was solubilized with 1% SDS and quantitated by scintillation counting; maximum binding ranged between 20 and 25% of input virus.

Virus growth and infectivity assays. Human rhinovirus type 3 (HRV3) was used as a prototype of a major receptor rhinovirus throughout this study because it has a higher affinity toward receptor than the more commonly used serotype, HRV14 (10). HRV3 (obtained from the American Type Culture Collection) and [^{35}S]methionine-labeled HRV3 were propagated in HeLa S3 cells and purified as described previously (11). [^3H]uridine-labeled HRV3 was prepared in the same manner as [^{35}S]methionine-labeled HRV3, except that infected cells were labeled in DMEM-2% fetal calf serum containing 1 μg of actinomycin C_1 and 100 μCi of [^3H]uridine per ml (Amersham). All infectivity assays were performed with HeLa S3 cells in DMEM-2% fetal calf serum. Viral plaque assays were performed by incubating various dilutions of HRV3 with monolayers of HeLa cells in 35-mm-diameter cluster wells (Costar) for 30 min at 34°C in a volume of 1 ml. The monolayers were then washed and overlaid with 0.5% agarose (SeaPlaque; FMC Corp.) in DMEM-2% fetal calf serum, and plaques were scored after 2 to 3 days of incubation at 34°C following staining with crystal violet (21). For measurement of infectivity at high multiplicity of infection (MOI) (see Fig. 3C), 0.1 ml of HRV3 at 10^7 PFU/ml was preincubated with various concentrations of soluble ICAM-1 for 30 min at 34°C and then added to wells of a 96-well microtiter dish containing 10^4 HeLa cells per well. The cultures were then scored after a single cycle of virus replication by staining with crystal violet (22) at 24 h postinfection and determining the optical density at 550 nm on a plate reader (Molecular Devices). All experiments were performed in triplicate, and the results were expressed as the concentration of ICAM-1 needed to reduce the optical density at 550 nm by 50%. Specific infectivity (PFU/cpm) was determined by plaque assay from the peak fractions of clearly resolved 149S, 135S, and 80S species.

Sedimentation analysis of rhinovirus. Samples in 0.1 ml

were sedimented through 5 ml of 5 to 25% sucrose gradients (in N buffer-1 mg of BSA per ml) at $225,000 \times g$ in an SW50.1 rotor for 45 min at 4°C . Gradients were fractionated from the bottom into approximately 20 fractions. Apparent *S* values were determined by the rate of sedimentation relative to standards (catalase, 11.3S; glutamate dehydrogenase, 22.6S; and rhinovirus, 149S).

Dot blot analysis of viral RNA. Peak fractions from a sucrose gradient were precipitated with 7% polyethylene glycol-0.6 M NaCl (21), and the pellets were resuspended in 0.1 ml of 1% SDS. RNase-free glycogen (Boehringer Mannheim) was added as a carrier to a concentration of 200 $\mu\text{g}/\text{ml}$, and RNA was extracted essentially as described by Rueckert and Pallansch (27) from equal amounts of [^{35}S]methionine radioactivity (2,500 cpm, or approximately 10 ng of HRV3). Samples were applied to Gene Screen Plus filters (NEN) in dot blot apparatus as described previously (31). Filters were then prehybridized with $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate)- $5\times$ Denhardt's solution-0.5% SDS-0.2 mg of salmon sperm DNA per ml for 1 h at 37°C and then hybridized with a γ - ^{32}P -labeled oligonucleotide probe for nucleotides 455 to 471 of the positive (+) strand of HRV14 (5'-GCATTCAGGGGCCGGAG-3'; final concentration of 2 ng/ml) for 18 h at 37°C . The blot was washed with $2\times$ SSC-1% SDS twice at 20°C and twice at 42°C before autoradiography.

RESULTS

Expression of soluble truncated forms of ICAM-1. ICAM-1 cDNAs encoding soluble proteins were constructed by inserting stop codons within the reading frame of the ICAM-1 polypeptide. Thus, stop codons were inserted immediately before the first residue of the transmembrane domain, at the predicted ends of domains 1, 2, and 3, domains 1 and 2, and domain 1 to produce a series of progressively truncated proteins (Fig. 1A). The cDNAs were cloned into the expression vector CDM8, transfected into COS cells for transient expression, and cotransfected with pSV2-dihydrofolate reductase into CHO cells for establishment of stable cell lines. In the experiment for which the results are shown in Fig. 1B, the secretion of various forms of truncated ICAM-1 in transiently transfected COS cells was examined. The results indicated that the entire extracellular domain, tICAM(453), and the two N-terminal domains, tICAM(185), were efficiently secreted from transfected COS cells as species of 80,000 Da and 43,000 Da, respectively (Fig. 1B). The level of expression of domains 1, 2, and 3 [tICAM(283)] was approximately 10-fold lower than those of the above-mentioned fragments, and the secreted protein was more heterogeneous in mobility on SDS-PAGE. Expression of domain 1 [tICAM(88)] could not be detected in COS cells, and alternative constructs in which the stop codon was shifted to several sites N or C terminal to residue 88 also failed to produce detectable amounts of protein. In order to obtain sufficient quantities of protein for functional and structural studies, CHO cell transfectants were prepared, cloned, and subjected to stepwise gene amplification in increasing concentrations of methotrexate. This resulted in the derivation of cell lines secreting 1.5 μg of tICAM(453) and 1.0 μg of tICAM(185) per ml. A stable cell line expressing tICAM(283) was not obtained, perhaps because the low level of secretion was at the limit of sensitivity of the immunoassay. The cells were adapted to serum-free media, and the secreted ICAM-1 proteins were purified to homogeneity from culture supernatants (Fig. 1C).

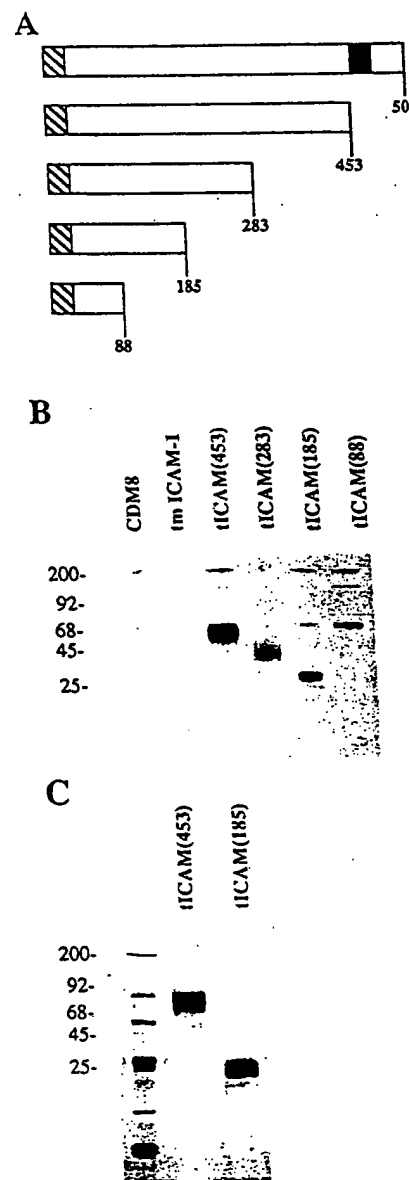


FIG. 1. Secretion of soluble ICAM-1 proteins. (A) Diagram of progressively truncated forms of ICAM-1 used in transfection experiments. The crosshatched section indicates signal sequence, and the filled section indicates the transmembrane region. (B) Fluorograph of [^{35}S]cysteine-labeled products secreted by COS cells analyzed by SDS-PAGE. The loading of lanes containing tICAM(283) and tICAM(88) is 10-fold higher than the loading of other lanes. (C) Silver-stained gel of purified tICAM(453) and tICAM(185) produced by CHO cell transfectants. In panels B and C, molecular weight markers (in thousands) are indicated on the left.

Several of the physical properties of tICAM(453) and tICAM(185) were examined. Both proteins were quantitatively immunoprecipitated by two monoclonal antibodies, c78.4 and c78.5, directed against two distinct conformation-dependent epitopes on ICAM-1 (data now shown), indicating that these epitopes were contained within the first two domains and providing evidence that the purified proteins were correctly folded. The Stokes radii of tICAM(453) and tICAM(185) were 5.3 and 3.9 nm, respectively, and the frictional ratio, f/f_0 (the ratio between the observed and

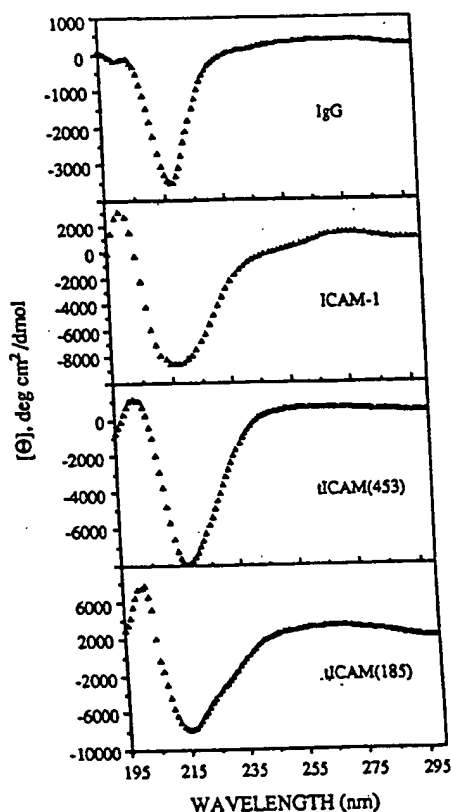


FIG. 2. CD spectra of IgG and ICAM-1 proteins. (A) c92.5 IgG; (B) ICAM-1; (C) tICAM(453); (D) tICAM(185). Data were collected and analyzed as described in Materials and Methods.

calculated frictional coefficients), was 1.9 for both tICAM(453) and tICAM(185), indicating that both fragments of ICAM-1 are highly asymmetric and elongated molecules. CD spectra were obtained for the soluble forms of ICAM-1. A single minimum at 210 to 220 nm is indicative of the presence of β structure and should be seen in proteins containing immunoglobulin-like domains because of the extensive amount of β structure in the immunoglobulin fold (38). As internal standards, spectra were collected for two members of the immunoglobulin supergene family of known 3-dimensional structure, β -2-microglobulin (4) and a murine monoclonal IgG (1). As expected, the spectra of β -2-microglobulin (not shown) and IgG (Fig. 2A) possessed single minima at 215 nm and 217 nm, respectively. The spectra of the two truncated proteins, tICAM(453) and tICAM(185), were similar to each other and to that of ICAM-1 (Fig. 2B, C, and D), with minima at 216 to 217 nm and a broad shoulder at 225 to 230 nm, although the shoulder was more pronounced in tICAM(185) than in tICAM(453). These CD spectra provide additional evidence that the soluble ICAM-1 proteins are properly folded and that they possess significant amounts of β structure.

Inhibition of virus binding by soluble ICAM-1. To compare the effects of the soluble ICAM-1 proteins on virus-receptor binding, a competition binding assay was employed. Various concentrations of soluble competitor ICAM-1 proteins were incubated with ^{35}S -HRV3, and binding to ICAM-1 immobilized on microtiter dishes was determined. As can be seen in Fig. 3A, both truncated forms of ICAM-1 inhibit HRV3 binding at similar concentrations: tICAM(453) and tICAM(185)

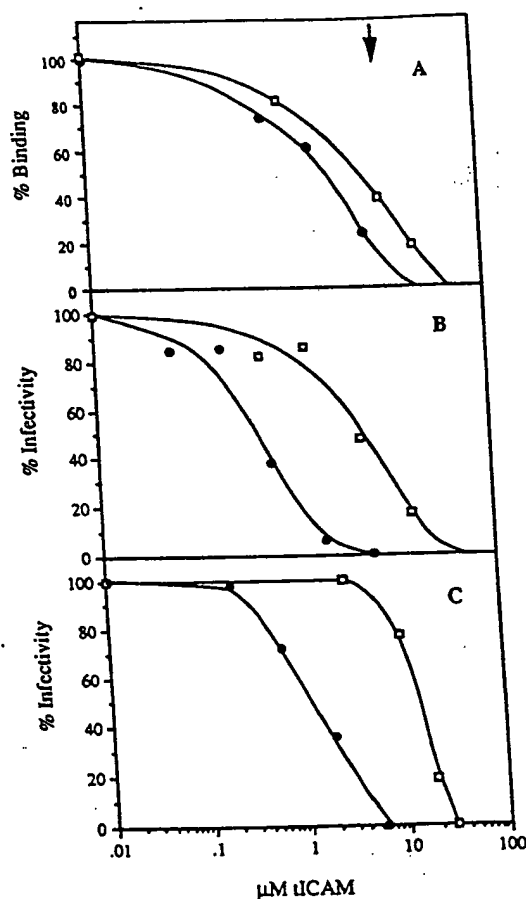


FIG. 3. Inhibition of virus binding and virus infectivity by soluble forms of ICAM-1. (A) ^{35}S -HRV3 was preincubated with various concentrations of soluble ICAM-1 for 30 min at 34°C, and binding to immobilized ICAM-1 was determined as described in Materials and Methods. (B) Reduction in virus titer by soluble ICAM-1. Approximately 2×10^2 PFU of HRV3 was preincubated with soluble ICAM-1 in 1 ml, and virus titer was determined by plaque assay as described in Materials and Methods. (C) Inhibition of virus infectivity under conditions of the virus-binding assay. HRV3 at 10^7 PFU/ml was preincubated with various concentrations of soluble ICAM-1 used to infect HeLa cells in a single-cycle, high-MOI infection (MOI, 100). Infectivity was determined after 24 h as described in Materials and Methods. tICAM(453), solid circles; tICAM(185), open squares. Arrow indicates the concentration of ICAM-1 at which the data shown in Fig. 4 were obtained.

at concentrations of $3.1 \pm 1.8 \mu\text{M}$ ($n = 3$) and $9.7 \pm 3.2 \mu\text{M}$ ($n = 3$), respectively, inhibit the binding of ^{35}S -HRV3 by 50%.

Inhibition of virus infectivity by soluble ICAM-1. The effect of soluble ICAM-1 on rhinovirus infectivity was examined under several different conditions (Fig. 3B and C; Table 1). In the experiment for which the results are shown in Fig. 3B and Table 1 (experiment I), HRV3 was preincubated with various concentrations of soluble ICAM-1, and virus titer was determined by a plaque assay in which soluble ICAM-1 was present during the incubation of the virus with the HeLa cell monolayers. The concentration of tICAM(185) required to reduce infectivity by 50% (IC_{50}) in this experiment was $5.3 \mu\text{M}$, similar to the concentration needed to inhibit virus binding by 50%. In contrast, the IC_{50} for infectivity of tICAM(453) was $0.4 \mu\text{M}$, eightfold lower than its IC_{50} for

TABLE 1. Neutralization of rhinovirus by soluble ICAM-1

Experiment ^a	Presence of ICAM-1 during:		IC ₅₀ (μM) ^b	
	Preincubation	Infection	tICAM(185)	tICAM(453)
I	+	+	5.3	0.4
II	+	+	13.2	1.2
III	+	+	>20	>20

^a Data for experiment I are shown in Fig. 3B, data for experiment II (high MOI) are shown in Fig. 3C, and data for experiment III are described in the text.

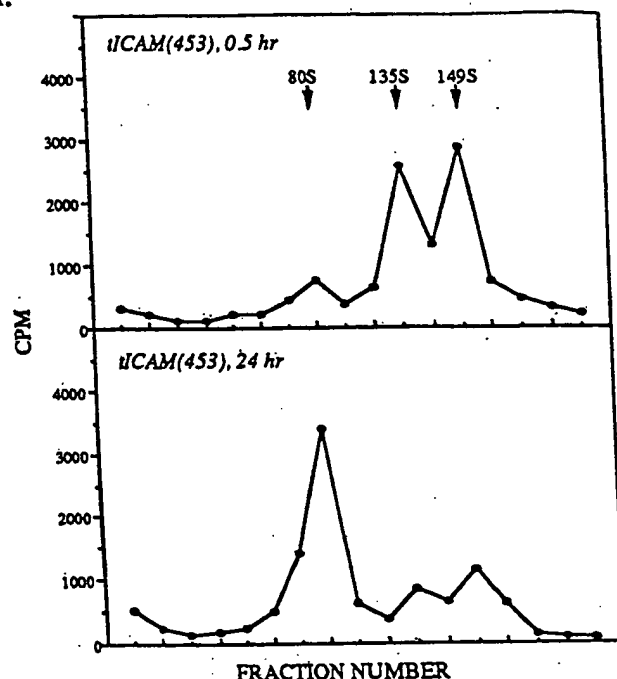
^b See Materials and Methods for a description of each assay.

virus binding. The IC₅₀ for infectivity of tICAM(453) was 13-fold lower than that of tICAM(185). Even though the binding experiments were performed in molar excess of ICAM-1 over receptor binding sites on rhinovirus (the concentration of HRV3 in these experiments was approximately 17 pM, and the concentration of potential receptor binding sites was 60-fold higher, or 1 nM), another infectivity experiment was performed in which the concentration of HRV3 was identical to that in the binding experiments. Consequently, HeLa cells were infected at a high MOI in the presence of soluble ICAM-1 in a single-cycle infection (Fig. 3C and Table 1 [experiment II]). Although the IC₅₀ values for tICAM(453) and tICAM(185) are approximately 3-fold higher in the high-MOI experiment than in the plaque assay, the relative difference in the IC₅₀ values between the two ICAM-1 species was approximately 11-fold, or essentially the same as in the low-MOI experiment. The difference in absolute IC₅₀ values between these two experiments may be due to a nonlinear relationship between infectious particles and infected cells at high MOIs. To determine if the neutralization of HRV3 was reversible, virus was preincubated with soluble ICAM-1 at 20 μM (a concentration known to reduce infectivity >99%; see Fig. 3B), and the mixture was then diluted to negligible ICAM-1 concentrations for infection of HeLa cells. The titers of tICAM(453)- and tICAM(185)-treated virus were 1.9×10^5 PFU/ml and 3.0×10^5 PFU/ml, respectively, compared with 3.6×10^5 PFU/ml for control virus (Table 1, experiment III). Only marginal reduction of virus titer (<50%) was observed at 20 μM soluble ICAM-1, indicating that the ICAM-1-mediated neutralization of rhinovirus is largely reversible; this reversibility is presumably due to simple dissociation of the virus-receptor complex upon dilution.

Thus, the neutralizing activity of tICAM(185) is directly correlated with its ability to inhibit virus-receptor binding, while tICAM(453) neutralizes rhinovirus at a concentration considerably lower than that necessary to inhibit binding and is presumably acting by a mechanism in addition to direct competition for receptor-binding sites on the virus. Neutralization of rhinovirus by both forms of ICAM-1 is largely reversible.

Effect of soluble ICAM-1 on rhinovirus integrity. Samples of ³⁵S-HRV3 incubated with tICAM(453) and tICAM(185) under conditions similar to those in the binding and infectivity experiments were analyzed by sedimentation through sucrose gradients. HRV3 incubated with 10 μM tICAM(453) separated into three peaks, 149S (cosedimenting with native virus), 135S, and 80S (Fig. 4A). The 149S and 135S peaks were infectious, with PFU/cpm ratios of 200 and 267 compared to a value of 200 for native HRV3. The specific infectivity of the 80S species was dramatically reduced to a

A.



B.

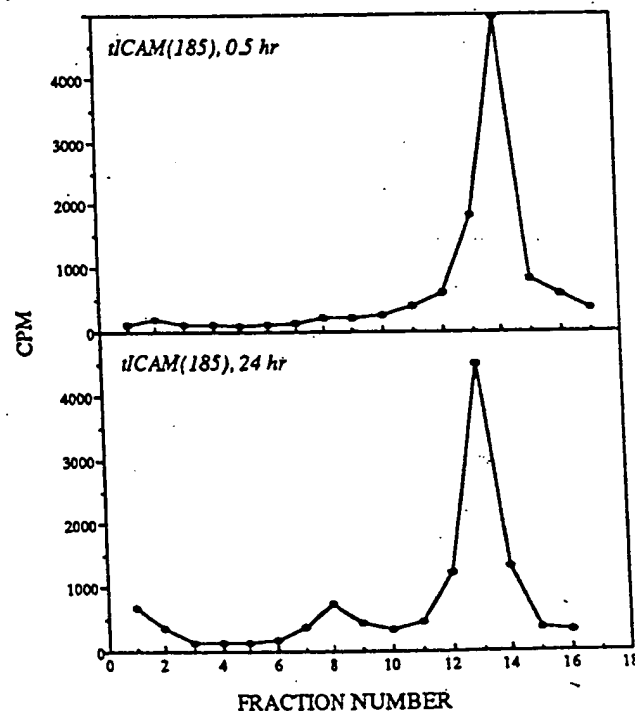


FIG. 4. Sedimentation analysis of HRV3 incubated with soluble ICAM-1. ³⁵S-HRV3 (10^5 cpm) was incubated with 10 μM tICAM(453) (A) or tICAM(185) (B) in N buffer-1 mg of BSA per ml (pH 7.5) for 0.5 and 24 h at 34°C. The mixtures were then sedimented through sucrose gradients as described in Materials and Methods, and the radioactivity in the fractions was determined by scintillation counting. Fractions are numbered from top to bottom of the gradient.

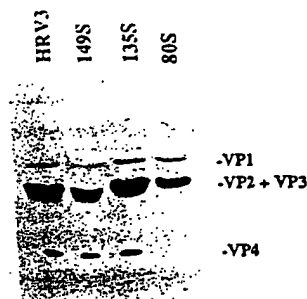


FIG. 5. Capsid protein composition of soluble ICAM-1-modified forms of HRV3. Fractions from control ^{35}S -HRV3 and 149S, 135S, and 80S [from ^{35}S -HRV3 incubated with $10\ \mu\text{M}$ tICAM(453)] were subjected to SDS-PAGE followed by fluorography. Densitometry (normalized to the VP1 band) indicated that the VP4 content of the 80S sample was 9% of control HRV3, 149S, or 135S species.

PFU/cpm ratio of 15; the residual infectivity is probably due to slight contamination from the 149S and 135S peaks since the gradients were fractionated from the bottom. This 80S species was also generated upon incubation with tICAM(185), although less efficiently. (Fig. 4B). The fraction of HRV3 in the 80S peak in the sample treated with $10\ \mu\text{M}$ tICAM(453) was 18% after 30 min and 61% after 24 h. The rate of conversion to the 80S species is highly temperature dependent and is greater at 37°C than at 34°C , the optimal temperature for rhinovirus growth (10). Since this concentration of tICAM(453) reduced infectivity to $<0.2\%$ (Fig. 3B) and since the reduction of infectivity is largely reversible, the conversion of rhinovirus to the noninfectious 80S species does not play a major role in rhinovirus neutralization by soluble ICAM-1.

The 149S, 135S, and 80S peaks were characterized with respect to their viral capsid composition and RNA content. Analysis of peak fractions by SDS-PAGE revealed that the 149S and 135S peaks possessed all four capsid proteins, while the 80S peak had dramatically reduced amounts of VP4 (Fig. 5). To assess the RNA content of the three peaks, preparations of [^{35}S]methionine- and [^3H]uridine-labeled HRV3 incubated with $10\ \mu\text{M}$ tICAM(453) were separated on sucrose gradients (Fig. 6). The radioactivity from the [^{35}S]methionine-labeled virus formed a clear 80S peak, which was insensitive to RNase A. The radioactivity from the [^3H]uridine-labeled virus formed 149S and 135S peaks but no 80S peak. When RNase A was included in the incubation, the 149S and 135S peaks were not altered, while the remainder of the radioactivity distributed across the top half of the gradient shifted to the top of the gradient. These data indicate that the 80S peak does not contain RNA and that the 149S, 135S, and 80S peaks are insensitive to RNase A digestion. In a second experiment, the viral RNA content was determined directly by extraction of RNA from equal amounts of radioactivity from 149S, 135S, and 80S peaks of [^{35}S]methionine-labeled HRV3 and then by dot blot analysis with an oligonucleotide probe for the positive (+) strand of rhinovirus (Fig. 7). This experiment showed that the 149S and 135S peaks but not the 80S peak contained viral RNA. Thus, the 80S peak appears to be an empty capsid, lacking both RNA and VP4. The 135S peak contains both VP4 and RNA and is infectious. Although the nature of the 135S peak is unclear at present, it is likely that it is a virion with altered hydrodynamic properties due to interaction with ICAM-1.

DISCUSSION

In this report, we have described the production and characterization of two soluble forms of ICAM-1, the major

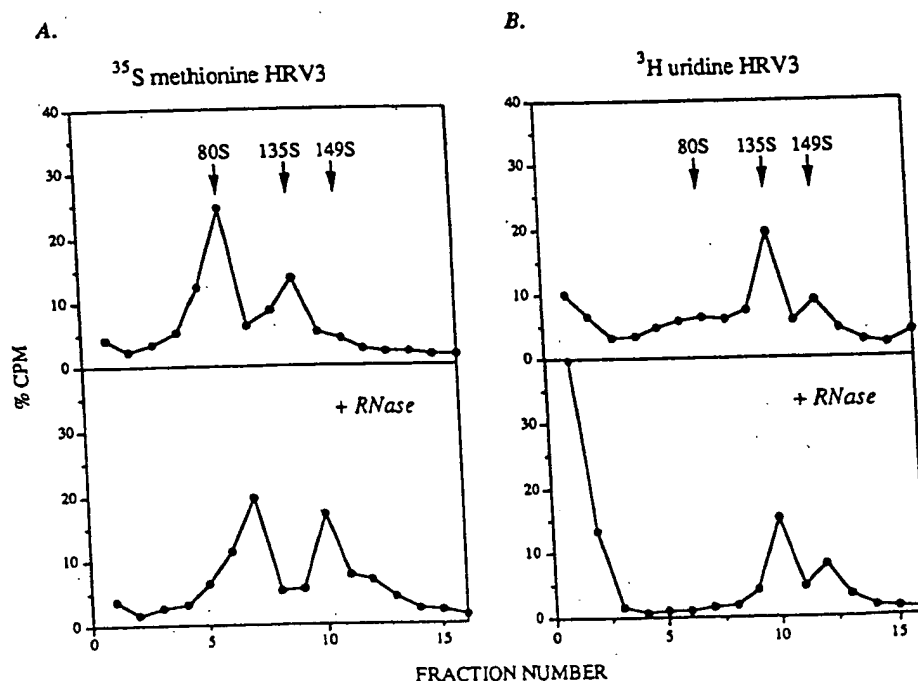


FIG. 6. Viral RNA content of soluble ICAM-1-modified forms of HRV3. Sedimentation analysis of [^{35}S]methionine- (A) and [^3H]uridine-labeled (B) HRV3 after incubation with $10\ \mu\text{M}$ tICAM(453) for 30 min at 37°C are shown. Where indicated, RNase A ($10\ \mu\text{g}/\text{ml}$) was included in the reaction mixtures. Data are plotted as the percentage of total radioactivity on each gradient (approximately 75,000 cpm and 60,000 cpm for [^{35}S]methionine- and [^3H]uridine-labeled HRV3, respectively). Fractions are numbered from top to bottom of the gradient.

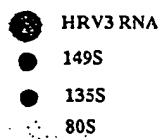


FIG. 7. Detection of viral RNA by hybridization with an oligonucleotide probe. RNA from the 149S, 135S, and 80S peaks of [32 S]methionine-labeled HRV3 incubated with tICAM(453) was probed with a [32 P]-labeled oligonucleotide probe for the positive (+) strand of rhinovirus. The amount of material loaded from each sample was normalized to the amount of [32 S]methionine radioactivity extracted, as described in Materials and Methods. HRV3 RNA (50 ng) and RNA from 8 ng (of protein) from the 149S, 135S, and 80S species were applied to the filter.

human rhinovirus receptor, and their effects on virus-receptor binding, virus infectivity, and virus integrity. The results from these experiments allow us to distinguish three distinct mechanisms by which soluble ICAM-1 blocks virus growth and to identify the regions of ICAM-1 responsible for these activities. In addition, the results presented show that receptor protein can uncoat the rhinovirus particle *in vitro* and, thus, have implications for the mechanism of virus uncoating *in vivo*.

ICAM-1 has a domain structure that is related to the immunoglobulin supergene family, and its extracellular portion can be divided into five immunoglobulin-like domains (32, 35). Two soluble forms of ICAM-1 have been produced, one corresponding to the entire extracellular domain [tICAM(453); domains 1 through 5] and one corresponding to the two N-terminal immunoglobulin-like domains [tICAM(185); domains 1 and 2]. Constructs coding for domains 1 through 3 were expressed poorly, and domain 1 was not expressed at all in mammalian cells. These data are similar to those obtained with CD4, a related member of the immunoglobulin supergene family (2). A likely explanation for these findings is that an intimate interaction exists between certain domains, particularly between domains 1 and 2, which is required for proper folding or solubility of the polypeptide; the close packing between domains 1 and 2 of CD4 revealed by its crystal structure has provided evidence in favor of this interpretation (28, 37). Physical characterization of tICAM(453) and tICAM(185) indicate that both are asymmetric molecules, consistent with the data of Staunton et al. (34) with regard to a soluble form of ICAM-1 that is essentially the same as tICAM(453). Proteins belonging to the immunoglobulin supergene family would be expected to have domains with the immunoglobulin fold motif, which is basically two apposed sheets of antiparallel β strands with connecting loops (38). CD indicates that both tICAM(453) and tICAM(185) have significant amounts of β structure, consistent with their sequence homologies to immunoglobulin supergene family members, and provides evidence that the proteins are properly folded. However, the CD spectra do have features that are significantly different from those of classical members of the immunoglobulin supergene family; the shoulder at 225 to 230 nm present in the two ICAM-1 species [particularly in tICAM(185)] is not found in the spectra of β -2-microglobulin and IgG. These differences suggest the presence of novel secondary structural features, particularly within domains 1 and 2. Indeed, the homology of many of the domains of ICAM-1 to immunoglobulin supergene family members determined by the ALIGN program (5), while significant, is not high (data not shown). Domain 1,

in particular, has a number of unusual features for an immunoglobulin-like domain, such as a relatively short distance (44 residues) between intradomain disulfide bonds and four instead of two cysteines in the B and F β strands. Thus, structural predictions based on the immunoglobulin fold motif should be made with caution.

Competitive binding studies indicate that the binding site for rhinovirus is largely contained within the first two domains. This conclusion is consistent with studies on transmembrane ICAM-1, in that human-mouse chimera studies by Staunton et al. (34) indicate that the rhinovirus binding site is located within domains 1 and 2. Similar studies by McClelland et al. (20) indicate that the rhinovirus binding site is located within domain 1. The reason for the small but significantly (threefold) greater inhibitory activity of tICAM(453) relative to that of tICAM(185) is unclear, although two possibilities are the fact that (i) tICAM(185) is less stably folded than tICAM(453) or (ii) the large size of tICAM(453) creates additional steric interference [relative to tICAM(185)] for a subsaturated virus particle binding to membrane-bound ICAM-1. In studies with transmembrane forms of ICAM-1, Staunton et al. (34) have reported that a shortened form of ICAM-1 containing only domains 1 and 2 binds rhinovirus at approximately 1/10 the level of full-length transmembrane ICAM-1, which they propose is due to inaccessibility to the receptor-binding site on the virus because of the short distance of the virus binding site of ICAM-1 from the plane of the membrane. Direct analysis of the stoichiometry of soluble ICAM-1-virus binding may help to resolve this issue.

The effects of tICAM(453) and tICAM(185) on virus infectivity are clearly distinguishable. tICAM(185) inhibits virus infectivity at essentially the same concentration at which it inhibits virus-receptor binding, indicating that its mode of action is by competitive inhibition of virus binding to cellular receptor. tICAM(453), however, inhibits infectivity at a concentration 10-fold lower than that required for inhibition of binding, indicating a second mechanism for neutralizing virus dependent upon functions encoded by domains 3, 4, and 5. Although the nature of this second mechanism is unclear at present, it is reasonable to conclude that entry or intracellular uncoating steps are involved. One possibility is that the large size of tICAM(453) relative to that of tICAM(185) creates steric problems for subsaturated virus-receptor complexes during the entry or uncoating steps; another possibility is that additional contacts with the virus or with adjacent tICAM(453) molecules on the virion mediated by domains 3, 4, or 5 are responsible for the enhanced neutralizing activity of tICAM(453). Marlin et al. (19) reported a similar disparity between the ability of a soluble ICAM-1 molecule similar to tICAM(453) to inhibit virus-receptor binding and virus infectivity, although this was attributed to differences in the experimental conditions of the two assays. In the binding and infectivity experiments reported here, soluble ICAM-1 is in considerable molar excess above that of virus or viral receptor binding sites and we have performed our binding and infectivity studies at identical rhinovirus concentrations. The results presented here indicate that the differences in the binding and neutralization activities are significant.

Both tICAM(453) and tICAM(185) have the ability to irreversibly inactivate rhinovirus by causing the loss of the viral subunit VP4 and the RNA genome; this constitutes a third mechanism of virus neutralization by soluble receptor. The subviral particles resulting from this alteration are similar in some respects to the eclipse products described for

poliovirus and other picornaviruses generated during infection of cells (7, 8, 13), which are thought to be products of the uncoating process (26). It has been demonstrated that poliovirus can be conformationally altered in cell-free systems by membranes containing poliovirus receptor (6, 12) or detergent-solubilized poliovirus receptor (14) to a 135S form lacking VP4 but still containing RNA (in an RNase-sensitive state). However, release of RNA requires further treatment of the 135S species with high concentrations of salt (14) or SDS (6, 12), and generation of 80S empty capsids requires live cells (8). We have demonstrated here that truncated soluble ICAM-1 can alter rhinovirus to an 80S species lacking both VP4 and RNA and, thus, essentially uncoats the virus. We have also identified an intermediate 135S particle which results from ICAM-1-rhinovirus interaction; this particle differs from the 135S poliovirus altered particle in that it is infectious and contains VP4. These differences between rhinovirus and poliovirus with respect to the products of *in vitro* virus-receptor interaction may reflect differences in the rate-limiting steps for uncoating between rhinovirus and poliovirus or may reflect the different experimental conditions under which the experiments were performed. The alteration of rhinovirus by soluble ICAM-1 clearly indicates that receptor can completely uncoat rhinovirus in the absence of other cellular components, suggesting that destabilization of the rhinovirus by receptor plays a role in the uncoating process *in vivo*. This may be a general phenomenon, as it has recently been reported that soluble CD4 induces the release of gp120 from the human immunodeficiency virus virion, and it has been hypothesized that this release of gp120 from virions attached to the cell surface may expose regions of gp41 molecules that could promote fusion of the virion and cell membranes (22, 23), which occurs at neutral pH at the cell surface (36). However, the physiological significance of ICAM-1-mediated uncoating *in vivo* is unclear since there is also a requirement for a chloroquine-sensitive low-pH step inside the cell for rhinovirus infection (10). A more detailed description of the *in vitro* alteration of rhinovirus by ICAM-1 and its role in virus uncoating *in vivo* will be presented elsewhere. However, it is clear from the data presented here that uncoating does not play a major role in soluble ICAM-1-mediated neutralization of rhinovirus under the conditions of optimal rhinovirus growth, since the inhibitory activity of soluble ICAM-1 toward virus infection is largely reversible and because the conversion to the 80S form can only account for a small fraction of this activity.

In conclusion, we have demonstrated that two forms of soluble ICAM-1, tICAM(453) and tICAM(185), inhibit virus-receptor binding and virus infectivity. Differential effects of these two proteins on these processes have defined three distinct mechanisms of virus neutralization. The first mechanism appears to be a simple competition of soluble receptor for receptor binding sites on the virus, and determinants within domains 1 and 2 of ICAM-1 are responsible for this activity. The second mechanism is a reversible neutralization in which virus is apparently blocked at an entry or uncoating step and involves contributions from domains 3, 4, and 5. The third mechanism is an irreversible inactivation of the virus characterized by the loss of the virus subunit VP4 and the RNA genome.

ACKNOWLEDGMENTS

We thank Judy Dziuba for tissue culture and fermentation, Tom Buckholz and Gary Davis for amino acid analysis, Craig Rice and George Mitra (Cutter Laboratories) for fermentation and purification of some of the tICAM(453) used in these studies, Suzy Pafka for

photography, David Osterman for instruction and discussions in the use of circular dichroism, Mark Cochran for helpful discussions, and Mike Kamarck for a critical reading of the manuscript.

REFERENCES

- Amzel, L. M., and R. J. Poljak. 1979. Three-dimensional structure of immunoglobulins. *Annu. Rev. Biochem.* 48:961-967.
- Arthos, J., K. C. Deen, M. A. Chaikin, J. A. Fornwald, G. Sathe, Q. J. Sattentau, P. R. Clapham, R. A. Weiss, J. S. McDougal, C. Pietropaolo, R. Axel, A. Truneh, P. J. Maddon, and R. W. Sweet. 1989. Identification of the residues in human CD4 critical for the binding of HIV. *Cell* 57:469-481.
- Bebbington, C. R., and C. C. Hentschel. 1987. The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells, p. 163-188. *In* D. M. Glover (ed.), *DNA cloning—a practical approach*, vol. 3. IRL Press, Oxford.
- Becker, J. W., and G. N. Reeke. 1985. Three dimensional structure of β 2-microglobulin. *Proc. Natl. Acad. Sci. USA* 82:4225-4229.
- Dayhoff, M. O., W. C. Barker, and L. T. Hunt. 1983. Establishing homologies in protein sequences. *Methods Enzymol.* 91:524-545.
- DeSena, J., and B. Mandel. 1977. Studies on the *in vitro* uncoating of poliovirus. II. Characteristics of the membrane-modified particle. *Virology* 78:554-566.
- Fenwick, M. L., and P. D. Cooper. 1962. Early interactions between poliovirus and ERK cells: some observations on the nature and significance of rejected particles. *Virology* 18:212-223.
- Ficks, C. E., and J. M. Hogle. 1990. Cell-induced conformational change in poliovirus: externalization of the amino terminus of VP1 is responsible for liposome binding. *J. Virol.* 64:1934-1945.
- Giranda, V. L., M. S. Chapman, and M. G. Rossmann. 1990. Modeling of the human intercellular adhesion molecule-1, the human major group receptor. *Proteins* 7:227-233.
- Greve, J. M. Unpublished data.
- Greve, J. M., G. Davis, A. M. Meyer, C. P. Forte, S. C. Yost, C. W. Marlor, M. E. Kamarck, and A. McClelland. 1989. The major human rhinovirus receptor is ICAM-1. *Cell* 56:839-847.
- Guttman, N., and D. Baltimore. 1977. A plasma membrane component able to bind and alter virions of poliovirus type 1: studies on cell-free alteration using a simplified assay. *Virology* 82:25-36.
- Joklik, W. K., and J. E. Darnell. 1961. The adsorption and early fate of purified poliovirus in HeLa cells. *Virology* 13:439-447.
- Kaplan, G., M. S. Freistadt, and V. R. Racaniello. 1990. Neutralization of poliovirus by cell receptors expressed in insect cells. *J. Virol.* 64:4697-4702.
- Kim, S., T. J. Smith, M. M. Chapman, M. G. Rossmann, D. C. Pevear, F. J. Dutko, P. J. Felock, G. D. Diana, and M. A. McKinlay. 1990. Crystal structure of human rhinovirus serotype 1A (HRV1A). *J. Mol. Biol.* 210:91-111.
- Kingston, R. E. 1987. Introduction of DNA into mammalian cells, p. 901-906. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. John Wiley & Sons, Inc., New York.
- Kishimoto, T. K., R. S. Larson, A. L. Corbi, M. L. Dustin, D. E. Staunton, and T. A. Springer. 1989. The leukocyte integrins. *Adv. Immunol.* 46:149-182.
- Marlin, S. D., and T. A. Springer. 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen (LFA-1). *Cell* 51:813-819.
- Marlin, S. D., D. E. Staunton, T. A. Springer, C. Stratowa, W. Sommergruber, and V. J. Merluzzi. 1990. A soluble form of intercellular adhesion molecule-1 inhibits rhinovirus infection. *Nature (London)* 344:70-72.
- McClelland, A., J. deBear, S. Connolly Yost, A. M. Meyer, C. W. Marlor, and J. M. Greve. 1991. Identification of monoclonal antibody epitopes and critical residues for rhinovirus binding in domain 1 of ICAM-1. *Proc. Natl. Acad. Sci. USA* 88:7993-7997.

21. Minor, P. D. 1985. Growth, assay, and purification of picornaviruses, p. 25-40. In B. W. J. Mahy (ed.), *Virology: a practical approach*. IRL Press, Oxford.
22. Moore, J. P., J. A. McKeating, W. A. Norton, and Q. J. Sattentau. 1991. Direct measurement of soluble CD4 binding to human immunodeficiency virus type 1 virions: gp120 dissociation and its implications for virus-cell binding and fusion reactions and their neutralization by soluble CD4. *J. Virol.* 65:1133-1140.
23. Moore, J. P., J. A. McKeating, R. Weiss, and Q. J. Sattentau. 1990. Dissociation of gp120 from HIV-1 virions induced by soluble CD4. *Science* 250:1139-1142.
24. Rossmann, M. G. 1989. The canyon hypothesis. Hiding the host cell receptor attachment site on a viral surface from immune surveillance. *J. Biol. Chem.* 264:14587-14590.
25. Rossmann, M. G., E. Arnold, J. W. Erikson, E. W. Frankenger, P. J. Griffith, H. Hecht, J. E. Johnson, G. Kamer, M. Luo, A. G. Mosser, R. R. Rueckert, B. Sherry, and G. Vriend. 1985. Structure of a common cold virus and functional relationship to other picornaviruses. *Nature (London)* 317:145-153.
26. Rueckert, R. R. 1990. Picornaviridae and their replication, p. 507-548. In B. N. Fields et al. (ed.), *Virology*. Raven Press, New York.
27. Rueckert, R. R., and M. A. Pallansch. 1981. Preparation and characterization of encephalomyocarditis (EMC) virus. *Methods Enzymol.* 78:315-326.
28. Ryu, S., P. D. Kwong, A. Truneh, T. G. Porter, J. Arthos, M. Rosenberg, X. Dai, N. Xuong, R. Axel, R. W. Sweet, and W. A. Hendrickson. 1990. Crystal structure of an HIV-binding recombinant fragment of human CD4. *Nature (London)* 348:419-426.
29. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239:487-491.
30. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning, a laboratory manual*. Cold Spring Laboratory Press, Cold Spring Harbor, N.Y.
31. Seed, B. 1987. An LFA-3 cDNA encodes a phospholipid-linked membrane protein homologous to its receptor CD2. *Nature (London)* 239:840-842.
32. Simmons, D., M. W. Makgoba, and B. Seed. 1988. ICAM, an adhesion ligand of LFA-1, is homologous to the neural cell adhesion molecule NCAM. *Nature (London)* 331:624-627.
33. Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature (London)* 346:425-434.
34. Staunton, D. E., M. L. Dustin, H. P. Erickson, and T. A. Springer. 1990. The arrangement of the immunoglobulin-like domains of ICAM-1 and the binding sites for LFA-1 and rhinovirus. *Cell* 61:243-254.
35. Staunton, D. E., S. D. Marlin, C. Stratowa, M. L. Dustin, and T. A. Springer. 1988. Primary structure of intercellular adhesion molecule 1 (ICAM-1) demonstrates interaction between the immunoglobulin and integrin supergene families. *Cell* 52:925-933.
36. Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman. 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. *Cell* 49:659-668.
37. Wang, J., Y. Yan, T. P. J. Garrett, J. Liu, D. W. Rodgers, R. L. Garlick, G. E. Tarr, Y. Husain, E. L. Rheinherz, and S. C. Harrison. 1990. Atomic structure of a fragment of human CD4 containing two immunoglobulin-like domains. *Nature (London)* 348:411-418.
38. Williams, A. F., and A. N. Barclay. 1988. The immunoglobulin superfamily-domains for cell surface recognition. *Annu. Rev. Immunol.* 6:381-405.

Herpes Simplex Virus-1 Entry into Cells Mediated by a Novel Member of the TNF/NGF Receptor Family

Rebecca I. Montgomery, Morgyn S. Warner,
Brian J. Lum, and Patricia G. Spear
Northwestern University Medical School
Department of Microbiology-Immunology
303 East Chicago Avenue
Chicago, Illinois 60611

Summary

We identified and cloned a cellular mediator of herpes simplex virus (HSV) entry. Hamster and swine cells resistant to viral entry became susceptible upon expression of a human cDNA encoding this protein, designated HVEM (for herpesvirus entry mediator). HVEM was shown to mediate the entry of several wild-type HSV strains of both serotypes. Anti-HVEM antibodies and a soluble hybrid protein containing the HVEM ectodomain inhibited HVEM-dependent infection but not virus binding to cells. Mutations in the HSV envelope glycoprotein gD significantly reduced HVEM-mediated entry. The contribution of HVEM to HSV entry into human cells was demonstrable in activated T cells. HVEM, the first identified mediator of HSV entry, is a new member of the TNF/NGF receptor family.

Introduction

Herpes simplex viruses (HSV) are human members of the neurotropic subgroup (alphaherpesviruses) of the herpesvirus family. Infections with HSV type 1 (HSV-1) and HSV type 2 (HSV-2) are highly prevalent. The usual manifestations of disease (reviewed by Corey and Spear, 1988) are mucocutaneous lesions of the mouth, face, eyes, or genitalia. Both HSV-1 and HSV-2 establish latent infections in neurons of peripheral ganglia and may reactivate to cause recurrent lesions. Rarely, the virus spreads to the central nervous system to cause meningitis or encephalitis.

Viral infection in the natural host usually remains localized to cells of the epidermis and peripheral nervous system, except in newborn infants, who are more prone to disseminated infection. Differentiated cell types probably vary in their susceptibility to HSV entry. However, cultured cells of various types from many animal species are susceptible to HSV infection, indicating that cell receptors for viral entry may be highly conserved or are multiple in number and are usually expressed on dividing cells.

The binding and penetration phases of HSV entry into cells can be experimentally dissociated. Binding of HSV-1 or HSV-2 to cells is mediated by interaction of viral envelope glycoproteins (gB or gC or both) with glycosaminoglycan chains (GAGs) of cell surface proteoglycans (reviewed by Spear, 1993). Penetration is by pH-independent fusion of the virion envelope with the cell plasma membrane or an early endosome (Wittels and Spear, 1991). Viral glycoproteins required for penetration include gB, gD, and gH-gL hetero-oligomers

(Sarmiento et al., 1979; Cai et al., 1988; Ligas and Johnson, 1988; Forrester et al., 1992; Roop et al., 1993). Binding of HSV to cells is not sufficient to mediate penetration. Certain cell types, such as swine testis (ST) or Chinese hamster ovary (CHO) cells, can bind virus efficiently but restrict viral entry (Shieh et al., 1992; Subramanian et al., 1994). Susceptible cells can be made resistant to HSV entry while retaining ability to bind virus by transfection with plasmids expressing HSV gD (Campadelli-Fiume et al., 1988; Johnson and Spear, 1989). These observations suggest that cell-associated gD may sequester or down-regulate a cellular protein required for HSV entry and that ST and CHO-K1 cells fail to express such a mediator of HSV entry.

By screening a human cDNA expression library for genes that could mediate HSV-1 entry into CHO-K1 cells, we identified a previously undescribed member of the tumor necrosis factor/nerve growth factor (TNF/NGF) receptor family, designated HVEM. This receptor mediated efficient entry of HSV-1 strains into CHO-K1 cells and ST cells and also enhanced the entry of an HSV-2 strain. HVEM was active in mediating HSV-1 entry into human T lymphoblasts.

Results

Cloning a Mediator of HSV-1 Entry

The CHO-K1 cell line is particularly resistant to entry of HSV-1 strain KOS (Shieh et al., 1992), abbreviated here KOS. A β -galactosidase-expressing version of this strain, HSV-1(KOS)gL86 abbreviated here KOS-gL86, was used to monitor viral entry. Production of β -galactosidase from the input viral genome signals that virus has entered the cell, released its genome to the nucleus, and activated the constitutive promoter driving β -galactosidase expression. Figure 1A shows that HeLa (human) cells were susceptible to infection by this virus, whereas CHO-K1 cells were highly resistant. Concentrations of input virus sufficient to infect 100% of HeLa cells left the CHO-K1 cells totally uninfected (Figure 1B).

The strategy to isolate a human cDNA that enhanced HSV-1 entry into CHO-K1 cells combined transient expression of cDNAs with KOS-gL86 challenge. Plasmid DNAs prepared from pools of a HeLa cell cDNA library were transfected into CHO-K1 cells. The cells were exposed to KOS-gL86 and then X-gal to identify infected cells. A pool containing a positive cDNA was subdivided by an iterative process to identify individual plasmid clones that had the desired phenotype.

A set of two plasmid clones (pBEC580 and pBEC748) that rendered transfected CHO-K1 cells susceptible to KOS-gL86 entry (Figures 1B and 1C) were isolated. Nucleotide sequencing of the cDNA insert (the same for both plasmids) revealed a 1698 bp cDNA encoding an open reading frame of 283 amino acids (Figure 2). The protein product, designated HVEM, has characteristics of a type I membrane glycoprotein with an N-terminal signal peptide, two potential sites for addition of N-linked glycans, and a probable membrane-spanning domain.

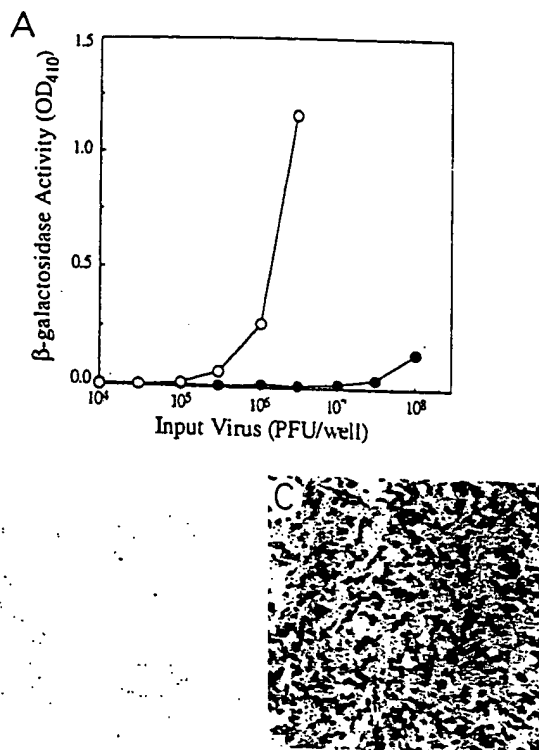


Figure 1. Entry of HSV-1 into HeLa Cells, CHO-K1 Cells, and Transfected CHO-K1 Cells

(A) CHO-K1 cells or HeLa cells were exposed to KOS-gL86 at the doses indicated. After the addition of virus (6 hr), infection was quantitated by monitoring the activity of β -galactosidase expressed from the input viral genome. In this and other figures, each point represents the mean of triplicate determinations. The individual values were within 10% of the mean. HeLa cells (open circles) and CHO-K1 cells (closed circles), values obtained 4 hr after ONPG addition.

(B and C) CHO-K1 cells were transfected with control plasmid pcDNA3 (B) or with pBEC580, isolated from the human cDNA library for its ability to confer susceptibility to HSV-1 infection (C). At 30 hr after transfection, the cells were exposed to KOS-gL86 at 100 pfu/cell. Later (6 hr) the cells were washed, fixed, and incubated with X-gal to identify infected cells.

Searches of protein databases with the predicted HVEM amino acid sequence failed to identify any proteins closely related to HVEM but did identify cysteine-rich repeats in HVEM that are characteristic of the TNF/NGF receptor family (Armitage, 1994). Pair-wise alignments of HVEM sequence with those of other human members of the receptor family revealed sequence identities ranging from 16.9% to 25.4% and similarities ranging from 26.9% to 37.1%. However, cysteines and other residues conserved within the cysteine-rich repeat regions of family members were also conserved in HVEM (residues marked with asterisks in Figure 2). Clearly, HVEM is a new member of the TNF/NGF receptor family.

Southern analyses were performed with HVEM probes and genomic DNAs from various cell lines. Probes from the purified cDNA hybridized to genomic DNA fragments from HeLa and HEP-2 (human) cells, Vero (monkey) cells, and CHO-HVEM12 cells (stably transfected with an HVEM cDNA clone), but not from control CHO-K1 cells (Figure 3). The smaller PvuII probe hybridized to a single

DNA fragment from three different human cell lines (HT1080 not shown) and to a single DNA fragment of different size from monkey cells (Figure 3B). The full-length EcoRI probe lacked sites for the restriction endonuclease used to digest the cell DNAs (BamHI) yet hybridized to additional bands in human and monkey DNA samples (Figure 3C). Thus, it is likely that HVEM is encoded by a single-copy human gene with multiple exons and that nonhuman primates have a related gene.

Expression of HVEM

Labeled probes prepared from HVEM cDNA were hybridized to a Northern blot of polyadenylated RNAs extracted from several human tissues. HVEM-related RNAs were detected in most samples tested, with highest levels in lung, liver, and kidney and least in brain (Figure 3E). The RNAs were heterogeneous in size. One species of about 2 kb was similar in size to the HVEM cDNA. The presence of larger species (4–5 kb) suggests that transcription of the gene for HVEM may result in multiple mRNAs.

We produced hybrid and epitope-tagged versions of HVEM to verify expression of the protein and to isolate material for immunization and detection of antibodies. HVEM:Fc, comprising the three-and-a-half cysteine-rich repeats of the ectodomain (see Figure 2) fused in-frame to the Fc region of rabbit IgG heavy chain, was secreted from transfected cells as a glycoprotein (about 50–65 kDa). Changes in size after treatment of HVEM:Fc with glycosidases were consistent with presence of O-linked and complex N-linked carbohydrate chains (Figure 4A). The glycosylation and secretion of HVEM:Fc suggests that the hydrophobic region between amino acids 23 and 38 serves as a signal sequence even though it does not strictly adhere to rules for cleavable signal sequences.

An epitope-tagged version of HVEM was also engineered by fusing HVEM cDNA encoding the first 257 amino acids (see Figure 2) to an oligonucleotide encoding an influenza virus hemagglutinin epitope (Flu epitope). CHO-K1 cells transfected with a plasmid expressing this protein (HVEM-257Flu) produced several Flu-tagged HVEM species ranging in size from about 30 to 90 kDa (Figure 4B). The higher molecular mass species may be multimers of HVEM-257Flu or have bulky posttranslational modifications.

Rabbits were immunized with purified HVEM:Fc and antiserum tested for antibodies specific for HVEM. Cell extracts from CHO-K1 cells transfected with control or HVEM-257Flu-expressing plasmids were immunoprecipitated with rabbit antiserum or control preimmune serum, followed by Western analyses for detection of the Flu epitope. The rabbit antiserum precipitated HVEM-257Flu (lanes 1 and 2) but not a Flu-tagged HSV-1 glycoprotein (lane 3), whereas preimmune serum lacked specific precipitating activity (Figure 4C). Anti-HVEM antibodies preferentially precipitated the higher molecular mass forms of HVEM-257Flu.

HVEM-Mediated Entry of HSV-1 and HSV-2 Strains into Transfected Cells

CHO-K1 and ST cell lines, both resistant to HSV-1 entry, were transfected with control or HVEM-expressing plasmids to obtain stable clones. The HVEM-expressing

```

1  CCTTCATACCGGCCCTTCCCCTCGGCTTTGCCTGGACAGCTCCTGCCTCCCGCAGGGCCACCTGTGTCCCC
73  CAGCGCCGCTCCACCCAGCAGGCTGAGCCCTCTCTGCTGCCAGACACCCCTGCTGCCACTCTCTGCT
145  GCTCGGGTTCTGAGGCACAGCTTGTACACCGAGGGGATTCTCTTTCTTCTCTTCTGGCCACAGCCG
217  CAGCAATGGCGCTGAGTTCTCTGCTGGAGTTTCATCTGCTAGCTGGGTCCCGAGCTGCCGGTCTGAGCCT
289  GAGGCATGGAGCCTCTGGAGACTGGGGCCTCTCCTGGAGATCCACCCAGAACCGACGTCTTGAGGC

1  M E P P G D W G P P P W R S T P R T D V L R
361  TGGTGTGTATCTCACCTTCTCTGGAGCCCCCTGCTACGCCCCAGCTCTGCCCTCTGCAAGGAGGACGAGT
23  L V L Y L T F L G A P C Y A P A L P S C K E D E
433  ACCCAGTGGGCTCCGAGTGTGCCCCAAGTGCAGTCCAGGTTATCGTGTGAAGGAGGCTGCGGGGAGCTGA
47  Y P V G S E C C P K C S P G Y R V K E A C G E L
505  CGGGCAGAGTGTGTGAACCTGCCCTCCAGGCACCTACATTGCCACCTCAATGGCCTAAGCAAGTGTCTGC
71  T G T V C E P C P P G T Y I A H L N G L S K C L
577  AGTGCCAAATGTGTGACCCAGCCATGGGCTGCGCGGACGCGGAAGTCTCCAGGACAGAGAAGCGCGTGT
95  Q C Q M C D P A M G L R A T R N C S R T E N A V
649  GTGGTGCAGCCAGGCCACTTCTGCATCGTCCAGGACGGGGACCACTGCGCGCGTGGCGCGTTACGCCA
119  C G C S P G H F C I V Q D G D H C A A C R R Y A
721  CCTCCAGCCCGGGCCAGAGGTGCAGAAGGGAGGCACGAGAGTCCAGGACACCTGTGTGCAAGTGCACCC
143  T S S P G Q R V Q K G G T E S Q D T L C Q N C P
793  CGGGGACCTTCTCTCCCAATGGGACCCTGGAGGAATGTGAGCACCAGACCAAGTGCAGCTGGCTGGTGACGA
167  P G T F S P N G T L E E C Q H Q T K C S W L V T
865  AGGCCGGAGCTGGGACAGCAGCTCCCACTGGGTATGGTGGTTTCTCTCAGGGAGCCTCGTCATCGTCATTG
191  K A G A G T S S S H W V W W F L S G S L V I V I
937  TTTGCTCCACAGTTGGCCTAATCATATGTGTGAAAGAGAAAGCCAAAGGGGTGATGTAGTCAAGGTGATCG
215  V C S T V G L I I C V K R R K P R G D V V K V I
1009  TCTCCGTCAGCGGAAAGACAGGAGGCAGAAGGTGAGGCCACAGTCATTGAGGCCCTGCAGGCCCTCCGG
239  V S V Q R K R Q E A E G E A T V I E A L Q A P P
1081  ACGTCACCACGGTGGCCGTGGAGGAGACAATACCCTCATTACGGGGAGGAGCCCAACCACTGACCCACAG
263  D V T T V A V E E T I P S F T G R S P N H -
1153  ACTCTGCACCCGACGCCAGATACCTGGAGCGACGGTGTCTGAAAGAGGCTGTCCACCTGGCGAAACCCAC
1225  CGGAGCCCGGAGGCTTGGGGCTCCGCTGGGTGGTCTCCGTCTCTCCAGTGGAGGGAGAGGTGGGGCC
1297  CCGTCTGGGGTAGAGCTGGGGACGCCACGTGCCATTCCATGGGCCAGTGGGGCTGGGGCTCTGTCTGTG
1369  CTGTGGCCTGAGCTCCCAAGAGTCTGAGGAGGAGCGCCAGTTGCCCTCGCTCACAGACCACACACCCAGC
1441  CCTCTGGGCCAGCCAGAGGGCCCTTCAGACCCAGCTGTCTGCGCGTCTGACTCTTGTGGCCTCAGCAGG
1513  ACAGGCCCGGGCACTGCCTCACAGCCAAAGCTGGAGTGGGTGGCTGAGTGTGGTGTAGTGGATACCA
1585  CATCGGAAGTGATTTCTAAATTGGATTGAATTCGGTCTCTCTATTGTCATGAAACAGTGTATTT
1657  GGGGAGATGCTGTGGGAGGATGTAATATCTTGTCTCTCAAAAAAAAAAAAAAAAAAAAAAAAAA

```

Figure 2. Nucleotide Sequence of the HVEM cDNA and Amino Acid Sequence of the Open Reading Frame. Features of the 283 amino acid open reading frame include a signal peptide (dotted underline), two potential sites for the addition of N-linked carbohydrate (single underline), and the probable membrane-spanning domain (double underline). Also indicated by patterned bars under the amino acid sequence are the three complete and one partial cysteine-rich repeats characteristic of members of the TNF/NGF receptor family. Asterisks show the positions of amino acids that are highly conserved in the family. The arrow after Cys-185 indicates the last amino acid of HVEM present in the fusion protein, HVEM:Fc. The arrow after Ala-257 indicates the last amino acid of HVEM present in the epitope-tagged truncated protein, HVEM257-Flu. The sequence deposited in GenBank was assigned accession number U70321.

clones were 100–1000 times more susceptible to KOS-gL86 infection than control clones (Figure 5A), which were as resistant as the parental cell lines. CHO-K1 cells, but not ST cells, are also resistant to entry of a porcine alphaherpesvirus, pseudorabies virus (PRV).

HVEM-expressing clones did not differ from control clones or parental cell lines in susceptibility to infection by a β -galactosidase-expressing mutant of PRV (data not shown). Thus, HVEM is not a general mediator of alphaherpesvirus entry.

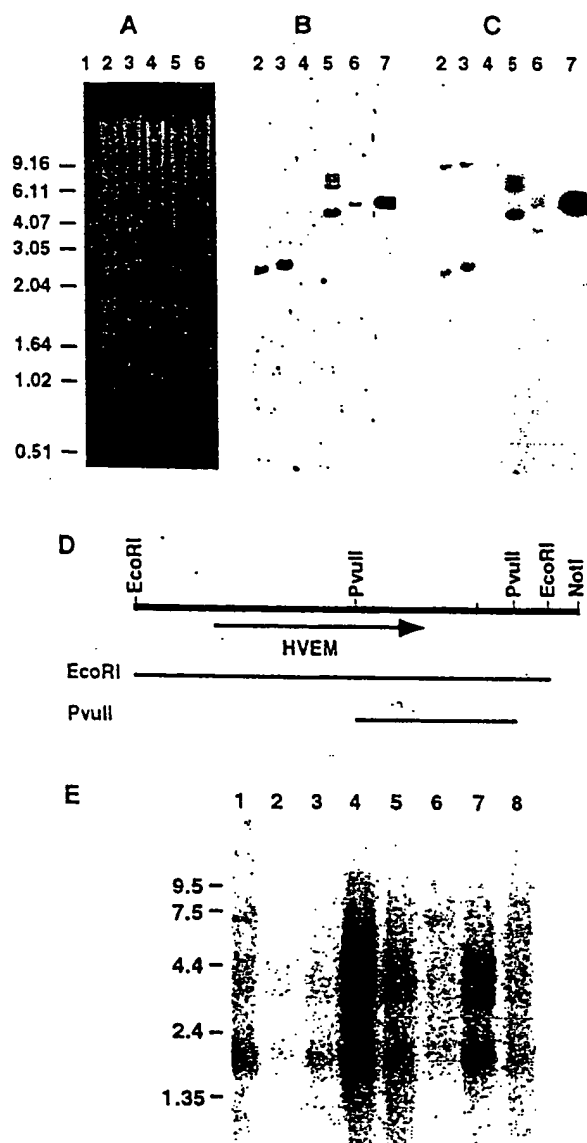


Figure 3. Southern and Northern Blots for Detection of HVEM-Homologous DNA and RNA in Cells and Tissues

(A–C) Cell DNAs were extracted and digested with BamHI and the fragments separated by electrophoresis and transferred to Duralon nylon membrane for hybridization. The DNAs were from HeLa (lanes 2), HEp-2 (lanes 3), CHO-K1 (lanes 4), CHO-HVEM12 (lanes 5), and Vero (lanes 6) cells. BamHI-digested plasmid, pBEC580, was also included (lanes 7).

(A) Photograph of the ethidium bromide-stained gel.

(B) Autoradiogram of the blot using the PvuII probe indicated in (D). (C) Autoradiogram of the blot using the EcoRI probe indicated in (D). (D) Schematic diagram of the HVEM cDNA and fragments used to generate probes. Numbers on the left of (A) and bands in lane 1 indicate molecular size markers (kbp).

(E) A Northern blot (Clontech) of polyadenylated RNAs extracted from various human tissues and hybridized with a 32 P-labeled probe from the PvuII fragment indicated in (D). The RNAs were extracted from heart (lane 1), brain (lane 2), placenta (lane 3), lung (lane 4), liver (lane 5), skeletal muscle (lane 6), kidney (lane 7), and pancreas (lane 8). Amounts on the blot were normalized with respect to actin mRNA content.

Infection of HVEM-expressing cells (CHO-HVEM12 and ST-HVEM1) by KOS-gL86 was inhibited in a dose-dependent manner by anti-HVEM serum or HVEM:Fc but not by preimmune serum or normal rabbit IgG (Figures 5B and 5C). In fact, preimmune serum reproducibly enhanced infection. As expected, neither anti-HVEM serum nor HVEM:Fc inhibited PRV infection of ST-HVEM1 cells (data not shown).

To determine whether the anti-HVEM serum inhibited HSV binding, CHO-HVEM12 cells were exposed to radio-labeled KOS at several virus concentrations to quantitate virus binding at 4°C in the presence of anti-HVEM serum or preimmune serum at a 1:90 dilution. This dilution of antiserum completely inhibited HSV-1 infection at all input doses of virus tested (Figure 5B; data not shown) but did not inhibit virus binding (data not shown). Thus, the antibodies blocked infection by interfering with penetration (or possibly with events leading to uncoating and expression of the genome). The antibodies did not render the cells unresponsive to virus entry in general or inhibit cell functions required for gene expression. Antibody-treated ST-HVEM1 cells remained fully susceptible to PRV entry and expressed β -galactosidase from the viral genome.

The ability of HVEM to mediate entry of other strains of HSV was determined. CHO-IE β 8 cells transfected with HVEM-expressing or control plasmid were challenged with various concentrations of different HSV-1 and HSV-2 strains. These cells contain the Escherichia coli *lacZ* gene downstream of an HSV-1 immediate-early promoter and express β -galactosidase upon viral entry and delivery of the HSV trans-inducer VP16 (Campbell et al., 1984; Batterson and Roizman, 1983) into the cell. Expression of HVEM significantly enhanced entry of all wild-type HSV-1 and HSV-2 strains tested (Figure 6).

CHO-IE β 8 cells transfected with control plasmid, like parental CHO-K1 cells, did not completely resist entry of HSV-1(MP) and HSV-2(333) (Figure 6). Some hamster factor probably mediated their entry, albeit inefficiently. HSV-2 strains in general infect CHO-K1 cells more efficiently than do HSV-1 strains (Shieh et al., 1992). HSV-1(MP) and HSV-1(KOS)804 are syncytial mutants with enhanced ability to induce cell fusion due to missense mutations in gK (Pogue-Geile and Spear, 1987; Roop et al., 1993). HSV-1(ANG) also has a syncytial phenotype due to a missense mutation in gB (Weise et al., 1987). Thus, the syncytial phenotype does not explain the enhanced ability of HSV-1(MP) to infect CHO cells.

Expression of HVEM failed to enhance the entry of HSV-1(KOS)rid1 (KOS-rid1), HSV-1(KOS)rid2 (KOS-rid2; data not shown), and HSV-1(ANG) (Figure 6). KOS-rid1 and KOS-rid2 are mutants selected for resistance to gD-mediated interference. Single amino acid substitutions in gD (Q27P or Q27R, respectively) account for the ability of these mutants to enter cells despite the block imposed by expression of wild-type gD by the cells (Dean et al., 1994). HSV-1(ANG) has the same amino acid substitution as KOS-rid2 (and other substitutions relative to KOS-gD) and is also resistant to gD-mediated interference (Dean et al., 1994). KOS-rid1 and KOS-rid2 are somewhat impaired, compared with the parental strain KOS, in ability to infect human Hep-2 cells, but they infect HEP-2 cells much more efficiently than CHO-K1



Figure 4. Expression of the HVEM:Fc Hybrid Protein and Epitope-Tagged HVEM-257Flu and Immunoprecipitation of HVEM-257Flu

(A) Samples of purified HVEM:Fc were appropriately treated for incubation with Endo F, Endo H, or Endo F/O-glycanase as indicated, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to nitrocellulose for Western blot analysis with anti-rabbit IgG peroxidase conjugates and substrate for chemiluminescent detection.

(B and C) CHO-K1 cells were transfected with two different subclones of pBEC14 for ex-

pression of HVEM-257Flu (lanes 1 and 2), with pMN114 for expression of a Flu-tagged truncated version of HSV-1 gL (lane 3), or with pCDNA3, a control plasmid (lane 4). At 48 hr after transfection, cell lysates were prepared and either immediately added to sample buffer for SDS-polyacrylamide gel electrophoresis (B) or mixed with preimmune or immune rabbit serum for immunoprecipitation, followed by solubilization of the immune precipitates for SDS-polyacrylamide gel electrophoresis (C). After electrophoresis, the separated proteins were transferred to nitrocellulose for Western blotting with the anti-Flu MAb 12CA5. Asterisks indicate the multiple Flu-tagged forms of HVEM detected. Numbers at the left of each panel indicate molecular mass markers in kDa.

cells (Dean et al., 1994). We conclude that viruses carrying the mutant (or ANG) form of gD cannot use HVEM as a mediator of entry but enter human cells by other pathways.

HVEM-expressing CHO cells were permissive for viral replication (data not shown), indicating that the principal block to replication in CHO-K1 cells is at the stage of virus entry. CHO-HVEM12 cells infected with KOS or HSV-1(F) produced 10,000-fold or 100-fold more progeny virus, respectively, than did control CHO-C8 cells or CHO-K1 cells, but 10-fold to 100-fold less than did HeLa cells.

HVEM-Mediated Entry of HSV into Human Cells

Use of the rabbit antiserum to detect HVEM on surfaces of human cells revealed that activated peripheral blood mononuclear cells expressed higher levels of HVEM than cell lines such as 293 and HeLa (C. Ware, R. I. M., and P. G. S., unpublished data). Phytohemagglutinin-activated T cell blasts (greater than 95% CD3+ and CD25+) were prepared and shown to express HVEM (Figure 7A). These cells were exposed to viruses expressing wild-type gD (KOS-gL86) or a mutant form of gD (HSV-1[KOS]rid1-tk12 [KOS-rid1-tk12], a β -galactosidase-expressing version of KOS-rid1) in the absence or presence of anti-HVEM serum or preimmune serum. In absence of either serum, the T cell blasts were more susceptible to infection with KOS-gL86 than with KOS-rid1-tk12 (Figure 7B). As was noted with the HVEM-expressing CHO and ST cells, anti-HVEM serum had a dose-dependent inhibitory effect on infection and pre-immune serum had a stimulatory effect, but only with KOS-gL86 (Figure 7B). Results similar to those shown in Figure 7 were obtained with activated T cell blasts from four different donors. We conclude that infection of T cell blasts with KOS-gL86 is largely HVEM-dependent.

Discussion

Demonstration that a new member of the TNF/NGF receptor family, HVEM, can mediate entry of HSV identifies another important family of cell-surface receptors used by viruses for entry. This adds to the growing body of evidence that multiple cell-surface components can be

required for each entry event. Adenoviruses bind to cells via fibers extending from vertices of the icosahedral virions through as yet undefined interactions. Entry is then facilitated by interaction of a protein at the base of each fiber with cell-surface integrins (Wickham et al., 1993). Human immunodeficiency virus binds to cells via interaction of gp120 with CD4 (Dalglish et al., 1984; Klatzmann et al., 1984; Maddon et al., 1988), but entry requires cofactor activity which, for different virus strains, can be provided by various members of the chemokine receptor family (Feng et al., 1996; Deng et al., 1996; Dragic et al., 1996; Choe et al., 1996; Doranz et al., 1996; Alkhatib et al., 1996). Similarly, HSV binds to cell-surface GAGs, but entry requires mediator activity, which was shown here to be provided by HVEM. It should be noted that both GAGs and a mediator of entry such as HVEM are required for HSV infection. Cells lacking GAGs (Shieh et al., 1992; Banfield et al., 1995) or a mediator can be 100–1000 times more resistant to infection than cells expressing both.

The stage of HSV entry at which HVEM operates must be either the membrane fusion reaction that occurs after binding of virus to cell-surface GAGs or the release of internal virion proteins, including VP16, from sites of entry and their transport to the cell nucleus. This follows from our findings that virus binding to CHO-K1 cells occurs efficiently in the absence of HVEM or in the presence of anti-HVEM antibody. Also, the ability of HVEM to enhance HSV entry can be detected in CHO-IE β 8 cells, which carry a reporter gene under control of an immediate-early HSV-1 promoter. In these cells, introduction of input viral VP16 into the cell nucleus suffices to induce β -galactosidase expression and score an entry event.

It seems likely that HVEM interacts with one or more of the virion envelope glycoproteins to trigger membrane fusion or induce postfusion uncoating events. The finding that HVEM:Fc can inhibit HSV infection is consistent with direct interaction with virion surface proteins. Candidate proteins include the four envelope glycoproteins (gB, gD, gH, and gL) required for HSV-1 entry but not for binding of virus to cells. Functional interaction of virions with HVEM is influenced by gD because amino acid substitutions in gD eliminated ability of HSV-1 to

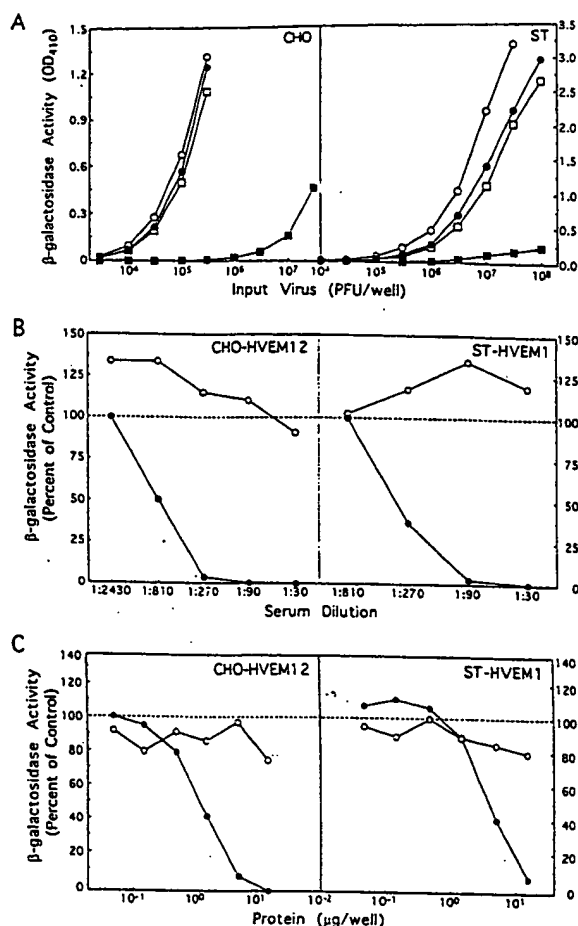


Figure 5. Enhanced Entry of HSV-1(KOS) into HVEM-Expressing Cell Lines and Inhibition of Infection by Anti-HVEM Antibodies or HVEM:Fc

(A) Several HVEM-expressing and control cell lines were obtained by transfection of CHO-K1 cells or ST cells with pBEC10 or control plasmid pcDNA3, followed by selection for stable maintenance of the plasmid. Representative clones were plated in 96 well plates and exposed to KOS-gL86 at the doses indicated. Later (6 hr), viral entry was quantitated as described in the legend to Figure 1. CHO-HVEM11 and ST-HVEM1 (open circles), CHO-HVEM9 and ST-HVEM22 (closed circles), and CHO-HVEM12 and ST-HVEM2 (open squares) were isolated after transfection with the HVEM-expressing plasmid pBEC10. CHO-C8 and ST-C8 (closed squares) were isolated after transfection with the control plasmid pcDNA3.

(B and C) CHO-HVEM12 cells or ST-HVEM1 cells were plated in 96 well dishes. In (B), the cells were exposed to preimmune or immune rabbit serum at the dilutions indicated for 30 min at 37°C. Various amounts of KOS-gL86 were then added in one-fifth volume, and incubation continued for 2 hr. In (C), virus was mixed with various concentrations of HVEM:Fc or normal rabbit IgG and incubated for 30 min at 37°C. The mixtures (50 μ l) were added to washed cells, and incubation continued for 2 hr. The virus-serum or virus-HVEM:Fc mixtures were then removed and the cells exposed briefly to low pH buffer to inactivate residual extracellular virus. The cells were washed and incubated for an additional 4 hr before lysis and addition of ONPG. The amount of virus added was 10^7 pfu/well in (B) and 10^4 pfu/well in (C). Open symbols were preimmune serum (B) or normal rabbit IgG (C). Closed symbols were immune serum (B) or HVEM:Fc (C).

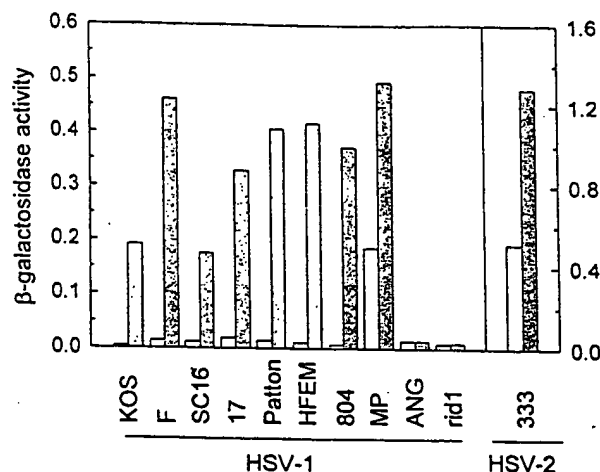


Figure 6. Enhanced Entry of HSV-1 and HSV-2 Strains into HVEM-Expressing CHO-IEB8 Cells

CHO-IEB8 cells were transfected with the HVEM-expressing plasmid pBEC10 (diagonally hatched bars) or the control plasmid pcDNA3 (open bars). At 24 hr after transfection, the cells were replated in 96 well dishes and, 24 hr later, exposed to various input doses of each of the virus strains indicated. Later (6 hr), the cells were lysed and ONPG added for the quantitation of viral entry. The results presented are for a single input dose of virus (30,000 pfu added per well) in the linear range of the dose response curve.

enter cells via HVEM (Figure 6) but not via other pathways of entry (Dean et al., 1994).

The role of HVEM in HSV entry could be completely dissociable from its normal physiological role. Ligand or ligands for HVEM and the consequences of ligand-receptor interaction remain to be identified. There is no obvious relationship between HSV envelope proteins and members of the TNF/NGF ligand family, although virions need not interact with HVEM in the same manner as its natural ligand. For members of the TNF/NGF receptor family, binding of ligand results in aggregation of receptors and activation of specific signal transduction pathways (Armitage, 1994; Heller and Krönke, 1994; Rothe et al., 1994). Binding of cognate receptors by members of the TNF or NGF family can have a variety of effects, including induction of cell proliferation, differentiation, or apoptosis, depending on the particular receptor, ligand, and other factors (Smith et al., 1994; Rabizadeh and Bredesen, 1994; Maness et al., 1994). Interestingly, another human herpesvirus, Epstein-Barr virus, expresses a membrane protein that interacts through a cytoplasmic domain with cytoplasmic proteins responsible for transmitting signals usually initiated by ligand binding to a TNF receptor family member (Mosialos et al., 1995). If any aspect of signaling through HVEM requires the C-terminal 26 amino acids, we can rule out its requirement for HSV entry based on the ability of HVEM-257Flu to mediate infection of CHO cells (data not shown).

Several lines of evidence indicate that other mediators of HSV entry have yet to be identified. First, although the cDNA for HVEM was isolated from a HeLa cell library, HVEM is probably not the principal mediator of HSV entry into HeLa cells. The anti-HVEM serum and HVEM:Fc had only marginal ability to block HSV-1 infection of HeLa cells (data not shown). Second, strains

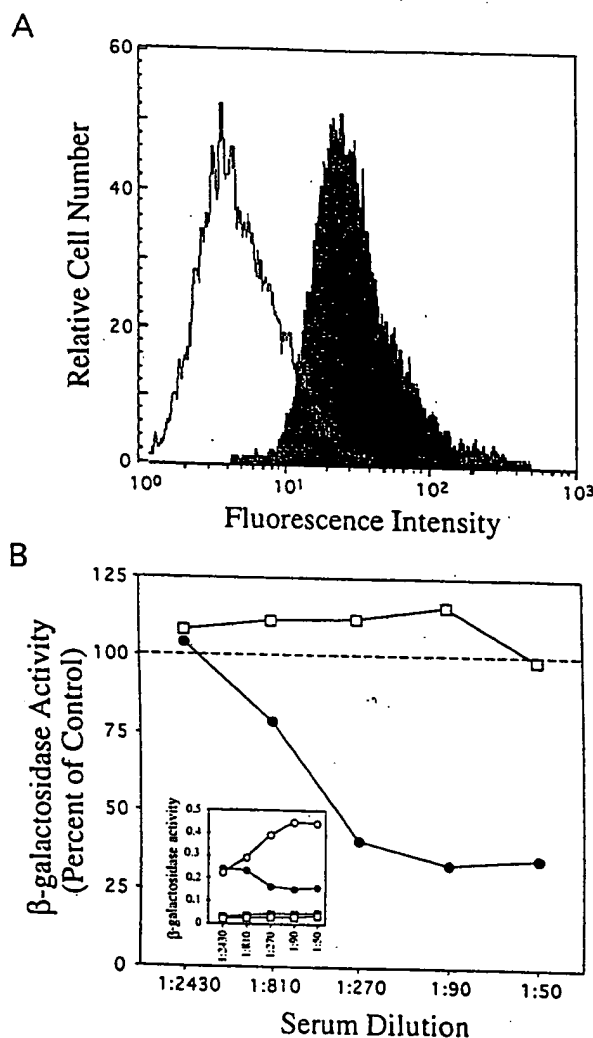


Figure 7. Expression of HVEM on Human T Lymphoblasts and HVEM-Dependent Infection by HSV-1 Expressing Wild-Type gD. (A) Activated T cells were incubated with anti-HVEM antiserum (shaded profile) or preimmune serum (open profile), followed by a fluoresceinated second antibody, and analyzed by flow cytometry. (B) Activated T cells were infected with KOS-gL86 or KOS-rid1-tk12 (10^4 pfu/well) in the absence or presence of anti-HVEM antiserum or preimmune serum. The cells were lysed and β -galactosidase activity quantitated at 6 hr after infection. The inset presents β -galactosidase activity expressed by KOS-gL86 (circles) or KOS-rid1-tk12 (squares) with preimmune (open symbols) or immune (closed symbols) serum. The large panel presents β -galactosidase activity in anti-HVEM-treated samples as a percentage of control values obtained with an equivalent concentration of preimmune serum, for KOS-gL86 (closed circles) and KOS-rid1-tk12 (open squares).

KOS-rid1, rid2, and HSV-1(ANG) can infect various human cell types but fail to infect HVEM-expressing CHO-K1 cells. Third, CHO-K1 cells express some factor that can mediate the entry of certain HSV strains, especially HSV-2 strains. Finally, continued screening of the HeLa cDNA library with other strains of HSV-1 indicates the presence of other genes that can mediate HSV entry (M. S. Warner, R. J. Geraghty, R. I. M., and P. G. S., unpublished data). These genes are not likely to encode basic fibroblast growth factor receptor, previously proposed to mediate HSV entry (Kaner et al., 1990), because

its expression in resistant cells did not enhance susceptibility to HSV entry (Shieh and Spear, 1991; Mirda et al., 1992). Mannose-6-phosphate receptors have also been proposed to mediate entry of HSV-1, but cell mutants lacking these receptors remain fully susceptible to viral entry (Brunetti et al., 1995). The effect of expression of these receptors in cells resistant to infection needs to be explored.

The fact that HVEM enhances entry of the wild-type HSV-1 and HSV-2 strains tested and mediates HSV entry into activated T cells highlights its probable importance in HSV pathogenesis. Several reports have described the replication of HSV in activated T cells (Pelton et al., 1977; Rinaldo et al., 1978; Teute et al., 1983) and the presence of infected lymphocytes in biopsies of cutaneous lesions (Boddingius et al., 1987). Because HVEM is a principal mediator of HSV entry into activated T cells, and subtle genetic alterations in HSV can eliminate ability to utilize HVEM as an entry mediator, virus strains in the field may differ in ability to infect activated T cells. The virulence of a virus strain is influenced by its ability to infect lymphocytes, whether this infection promotes virus spread or abrogates localized immune responses. Discovery of HVEM opens new areas of investigation, including exploration of factors that induce HVEM expression in lymphocytes and other human cells, potential role of signal transduction in viral entry or viral replication, and effects of human or viral genetic polymorphisms on HVEM-virus interactions.

Experimental Procedures

Cells and Viruses

CHO-K1, HEP-2, HeLa, Vero, and ST cells were obtained from the American Type Culture Collection and HT1080 cells from Dr. N. Bouck (Northwestern University). The CHO-IE β 8 cell line was isolated after transfection of CHO-K1 cells with pMLP01, a plasmid having the E. coli *lacZ* gene under control of the HSV-1 ICP4 promoter and expressing β -galactosidase upon infection of cells with HSV (M. L. Parish, R. I. M., and P. G. S., unpublished data).

Wild-type virus strains used were HSV-1(KOS), HSV-1(HFEM), HSV-1(Patton), HSV-1(F) (Ejercito et al., 1968), HSV-1(SC16), HSV-1(17) (McGeoch et al., 1988), and HSV-2(333). Mutant strains included KOS-rid1 and rid2 (Dean et al., 1994), HSV-1(KOS)804 (Little and Schaffer, 1981), HSV-1(MP) (Hoggan and Roizman, 1959), and HSV-1(ANG) (Munk and Donner, 1963; Weise et al., 1987; Dean et al., 1994). KOS-rid1-tk12 is a recombinant virus produced by inserting the E. coli *lacZ* gene driven by the HSV-1 ICP4 promoter in place of the thymidine kinase gene of KOS-rid1. These strains were propagated by passage on HEP-2 cells and titered on Vero cells. KOS-gL86, a mutant in which the E. coli *lacZ* gene with CMV promoter replaces part of the gL open reading frame (M. J. Novotny and P. G. S., unpublished data), was propagated and titered on gL-expressing Vero cell transfectants. A PRV mutant, in which the gH gene was interrupted by the *lacZ* gene (Klupp et al., 1994), was obtained from T. C. Mettenleiter and propagated and titered on gH-expressing Vero cell transfectants. Mutant viruses obtained from these complementing cell lines were fully infectious for and expressed β -galactosidase in noncomplementing cells but produced only noninfectious virus.

Infectivity Assays

Infectivity assays were based on quantitation of β -galactosidase expressed from the viral genome or by the β -galactosidase-expressing cell line CHO-IE β 8. Adherent cells were plated in 96 well tissue culture dishes ($2-4 \times 10^4$ cells/well) at least 16 hr prior to infection. Cells were washed and exposed to virus (in 50 μ l of phosphate-buffered saline [PBS] containing glucose and 1% calf serum

(PBS-G-CSF) for 6 hr at 37°C before solubilization in 100 μ l of PBS containing 0.5% NP-40 and the β -galactosidase substrate, o-nitrophenyl β -D-glucopyranoside (ONPG, 3 mg/ml). The reaction was monitored by spectrometry at several timepoints after the addition of ONPG to define the interval over which the generation of product was linear with time (Dynatech ELISA reader or a Spectromax 250). Alternatively, cells plated in 6 well tissue-culture dishes were exposed to virus and infected cells visualized using the β -galactosidase substrate X-gal (GIBCO Laboratories), which yields an insoluble blue reaction product. After infection, washed cells were fixed (PBS containing 2% formaldehyde and 0.2% glutaraldehyde), permeabilized (2 mM MgCl₂ containing 0.01% deoxycholate and 0.02% NP-40), and incubated with buffered X-gal (0.5 mg/ml). Antibody inhibition assays were performed as described for infectivity assays in 96 well plates, except that rabbit serum diluted in PBS-G-CSF was added to the cells (50 μ l/well) 30 min before addition of virus (10 μ l/well). After 2 hr with the serum-virus mixtures, the cells were treated with 0.1 M citrate buffer (pH 3.0) for 1 min to inactivate extracellular virus (Huang and Wagner, 1964; Highlander et al., 1987). The cells were then washed and incubated in PBS-G-CSF for 4 hr before solubilization and quantitation of β -galactosidase activity. Assays for inhibition of infection by HVEM:Fc were similar, except that dilutions of virus were mixed with HVEM:Fc or rabbit IgG and incubated for 30 min at 37°C prior to addition of the mixtures (50 μ l/well) to washed cell monolayers.

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation of freshly collected heparinized blood and incubated 3 days at 10⁶ cells/ml in RPMI-1640 containing 10% fetal bovine serum and purified phytohemagglutinin (Sigma Chemicals) at 5 μ g/ml. T cell blasts were isolated on 50% Percoll cushions and were routinely more than 95% CD3+ and more than 98% CD25+. HVEM expression was determined by incubating cells with the anti-HVEM rabbit serum or preimmune serum, followed by goat anti-rabbit fluoresceinated secondary antibodies and flow cytometry (Beckton-Dickenson FACScan). Remaining cells were dispensed into 96 well plates (10⁵ cells/well) for infectivity studies. Assays were as described above except that after 2 hr of incubation, the virus inocula were removed and replaced with medium containing preimmune or immune serum at the same concentrations as present during virus inoculation.

The binding of virus to cells was quantitated as described previously (Shieh et al., 1992) using ³H-thymidine-labeled purified HSV-1(KOS).

Isolation and Sequencing of the HVEM cDNA

A unidirectional HeLa cell cDNA expression library cloned into pcDNA1 in *E. coli* (Invitrogen) was plated onto 100 150 mm Luria-Bertani plates containing appropriate drugs (1.5 \times 10⁵ bacteria/plate). Colonies were pooled by scraping and frozen as 100 glycerol stocks. Samples of each stock were combined into groups of 10 and grown to stationary phase in broth. Plasmids prepared from each culture were transfected into CHO-K1 cells using LipofectAMINE (GIBCO Laboratories; 1.5 μ g of plasmid and 5 μ l of LipofectAMINE/35 mm culture). For controls, cells were transfected with pMN84, a plasmid expressing β -galactosidase, or were incubated with LipofectAMINE alone. At 30 hr after transfection, the cells were washed, inoculated with KOS-gL86 at about 100 pfu per cell, and then stained with X-gal as described above. Transfection efficiencies ranged from 30%–55% of cells based on expression of β -galactosidase from pMN84 in uninfected cells. In the first round of screening the cDNA library, plasmids from one group of 10 bacterial stocks from the library converted about 20–30 cells in the monolayer to susceptibility to KOS-gL86 infection. The frequency of conversion to susceptibility was about 10 times higher for one of the 10 stocks in this group. This stock was divided again into 100 pools, and, by an iterative process, two bacterial clones were obtained that yielded plasmids (pBEC580 and pBEC748) with the desired phenotype.

Both strands of the cDNA insert of pBEC580 were sequenced using Sequenase (Amersham) and T7 and Sp6 primers, as well as other primers generated as sequence was obtained. All primers were obtained from the Northwestern University Biotechnology Center. Single-strand sequencing of pBEC748 revealed the insert to be the same as that of pBEC580.

Southern and Northern Blots

Genomic DNAs were isolated (Hirt, 1967), digested with BamHI, and electrophoresed on 0.8% agarose gels. Southern blots were performed by standard procedures. Briefly, DNA was transferred to Duralon nylon membrane. Randomly primed probes were generated using digoxigenin-labeled nucleotides (Genius Kit; Boehringer Mannheim) or ³²P-labeled nucleotides. Prehybridization was for 2 hr in hybridization buffer with Denhardt's reagent and 50% formamide. After hybridization with probe at 42°C for 24 hr, membranes were incubated twice for 15 min at 65°C with 0.2 \times SSC containing 0.1% SDS. The blot was exposed to X-ray film at –70°C (for ³²P-labeled probes) or processed for digoxigen detection by chemiluminescence (Boehringer Mannheim), utilizing the alkaline phosphatase substrate CDP-STAR™ (Tropix).

Northern analysis was performed using a commercial blot of polyadenylated RNAs extracted from various human tissues (Clontech). Prehybridization and hybridization of the blot were done as described above at 42°C and washes with 0.2 \times SSC containing 0.1% SDS were at 50°C. The blot was exposed to X-ray film at –70°C.

Plasmids and Stably Transfected Cell Lines

Plasmid pBEC10, carrying the HVEM insert and a neomycin-resistance gene, was generated by cloning a HindIII–XhoI fragment of pBEC580 into pcDNA3. pBEC14, expressing HVEM-257Flu, was generated in several steps. The HindIII-to-SfiI fragment of the HVEM insert from pBEC580 was modified by deletion between the BamHI and BstYI sites, then inserted between HindIII and EcoRI sites of pMN104 (blunt-end ligation between the SfiI and EcoRI sites after Klenow treatment). pMN104 contains an oligonucleotide, inserted between the EcoRI and XbaI sites of pcDNA3, that encodes 11 amino acids (EFYPYDVPDYASL) plus a stop codon, including a 9 amino acid Flu epitope (underlined, Wilson et al., 1984). pMN114, also derived from pMN104, expresses a truncated Flu-tagged version of HSV-1 gL (M. J. Novotny and P. G. S., unpublished data). pBL58, expressing a hybrid form of HVEM (the ectodomain fused to the hinge, C₁2 and C₁3 domains of the rabbit IgG heavy chain), was generated in several steps. It consists of a cytomegalovirus promoter from pcDNAneo (SpeI to HindIII); HindIII to XbaI from pGEM3; the ectodomain of HVEM from pBEC580 (NheI site to a PvuII site just downstream of the last Cys residue); a fragment of rabbit IgG heavy chain cDNA from plasmid 3-4 (obtained from K. Knight at Loyola University Medical Center) including an EcoRI site added by polymerase chain reaction 5' to the rabbit sequence ACAAGACCGTTC and extending to a PstI site downstream of the reading frame (after cleavage with EcoRI, the filled-in site was blunt end-ligated to the PvuII end of the HVEM fragment); and HindIII to NheI from pGEM4 (the PstI end of the rabbit sequence was blunt end-ligated to the HindIII site, and the NheI site was ligated to the SpeI site of the CMV promoter fragment).

Stable HVEM-expressing cell lines were produced by transfection of CHO-K1 cells and ST cells with pBEC10 and selection in medium containing Geneticin (500 μ g/ml for CHO-K1 cells and 800 μ g/ml for ST cells). Surviving cells were cloned by limiting dilution, and cell clones susceptible to KOS-gL86 infection were subcloned and expanded. Control cell lines were produced by transfecting CHO-K1 and ST cells with pcDNA3 and isolating Geneticin-resistant clones.

HVEM:Fc Production, Characterization, and Use as Immunogen

HVEM:Fc secreted into the medium of pBL58-transfected CHO-K1 cells was purified by Protein G-Sepharose chromatography. HVEM:Fc was denatured by boiling for 5 min in 0.5% SDS, 1% β -mercaptoethanol, and carbohydrate modifications determined by incubating overnight at 37°C with endo F (200 mU, 1% NP-40, 50 mM sodium phosphate [pH 7.5]) or endo H (1 mU, 50 mM sodium citrate [pH 5.5]) or without added enzyme (1% NP-40, 50 mM sodium phosphate [pH 7.5]). Alternatively, samples were digested overnight at 37°C with neuraminidase (4 mU, 50 mM sodium citrate [pH 4.5]), then denatured and incubated overnight at 37°C with endo F (200 mU) and O-glycosidase (0.5 mU) in 1% NP-40, 50 mM sodium phosphate (pH 7.5). Western blots of control and glycosidase-treated samples were probed with a mixture of anti-rabbit IgG peroxidase conjugates (GibcoBRL 9814SA and Sigma A6667) at concentrations

of 1:1000 in BLOTTO (10 mM Tris [pH 7.4], 150 mM NaCl, 5% powdered milk, 0.05% Tween-20), followed by chemiluminescent detection with ECL reagent and Hyperfilm-MP (Amersham).

Rabbit polyclonal antibodies were produced by subcutaneous injection of purified HVEM:Fc mixed with Hunter's TiterMax adjuvant at Pocono Farms, Inc. For immunoprecipitation, lysates were prepared with 1% Triton X-100 in 150 mM NaCl, 20 mM Tris-HCl (pH 8.0), containing protease inhibitors (2 µg/ml of aprotinin; 2 µg/ml of leupeptin; 1 µg/ml of pepstatin A; 5 mM phenylmethylsulfonyl fluoride) and mixed on ice with rabbit preimmune or immune serum (10 µl/200 µl of lysate). Samples of cell lysates and immunoprecipitates collected on Protein A-agarose were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated in BLOTTO for 1 hr of blocking and then with the anti-hemagglutinin antibody 12CA5 (Wilson et al., 1984) diluted 1:5000 in BLOTTO, followed by horseradish peroxidase-coupled goat anti-mouse IgG (Boehringer Mannheim) diluted 1:10,000. Detection of second antibody was by incubation in ECL reagent and exposure to Amersham Hyperfilm-MP (Amersham).

Acknowledgments

We gratefully acknowledge the following contributions. M. J. Novotny produced KOS-gL86 and several plasmids used in this study. M. L. Parish collaborated in production of the CHO-IEß8 cell line and analysis of antibodies. N. Susmarski provided invaluable assistance with cell lines and virus stocks, and K. Coleman assisted with nucleotide sequence analysis. We thank C. Ware for permission to describe unpublished results obtained with the anti-HVEM antibodies, G. Kansas for advice and assistance with the activated T cell experiments, and C. Waltenbaugh for use of his spectrometer. We also thank N. Bouck, K. Knight, E. Mocarski, and T. C. Mettenleiter for gifts of materials. This work was supported by a grant from the National Institute of Allergy and Infectious Diseases (R01 AI 36293). R. I. M. is supported by National Research Service Award F32 AI09022.

Received June 5, 1996; revised September 23, 1996.

References

Alkhatib, G., Combadiere, C., Broder, C.C., Feng, Y., Kennedy, P.E., Murphy, P.M., and Berger, E.A. (1996). CC CKR5: A RANTES, MIP-1 α , MIP-1 β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 272, 1955-1958.

Ammitage, R.J. (1994). Tumor necrosis factor receptor superfamily members and their ligands. *Curr. Opin. Immunol.* 6, 407-413.

Banfield, B.W., Leduc, Y., Esford, L., Schubert, K., and Tufaro, F. (1995). Sequential isolation of proteoglycan synthesis mutants by using herpes simplex virus as a selective agent: evidence for a proteoglycan-independent virus entry pathway. *J. Virol.* 69, 3290-3298.

Batterson, W., and Roizman, B. (1983). Characterization of the herpes simplex virion-associated factor responsible for the induction of a genes. *J. Virol.* 46, 371-377.

Boddingius, J., Dijkman, H., Hendriksen, E., Schiff, R., and Stolz, E. (1987). HSV-2 replication sites, monocyte and lymphocytic cell infection and virion phagocytosis by neutrophils, in vesicular lesions on penile skin. *J. Cutan. Pathol.* 14, 165-175.

Brunetti, C.R., Burke, R.L., Hoflack, B., Ludwig, T., Dingwell, K.S., and Johnson, D.C. (1995). Role of mannose-6-phosphate receptors in herpes simplex virus entry into cells and cell-to-cell transmission. *J. Virol.* 69, 3517-3528.

Cai, W., Gu, B., and Person, S. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. *J. Virol.* 62, 2596-2604.

Campadelli-Fiume, G., Arsenakis, M., Farabegoli, F., and Roizman, B. (1988). Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. *J. Virol.* 62, 159-167.

Campbell, M.E.M., Palfreyman, J.W., and Preston, C.M. (1984). Identification of herpes simplex virus DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription. *J. Mol. Biol.* 180, 1-19.

Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., and Sodroski, J. (1996). The β -chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85, 1135-1148.

Corey, L., and Spear, P.G. (1988). Infections with herpes simplex viruses. *N. Engl. J. Med.* 314, 686-689; 749-757.

Dalglish, A.G., Beverley, P.C.L., Clapham, P.R., Crawford, D.H., Greaves, M.F., and Weiss, R.A. (1984). The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* 312, 763-766.

Dean, H.J., Terhune, S., Shieh, M.-T., Susmarski, N., and Spear, P.G. (1994). Single amino acid substitutions in gD of herpes simplex virus 1 confer resistance to gD-mediated interference and cause cell type-dependent alterations in infectivity. *Virology* 199, 67-80.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R., and Landau, N.R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381, 661-666.

Doranz, B.J., Rucker, J., Yi, Y., Smyth, R.J., Samson, M., Peiper, S.C., Parmentier, M., Collman, R.G., and Doms, R.W. (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the β -chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* 85, 1149-1158.

Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P., and Paxton, W.A. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 381, 667-673.

Ejercito, P.M., Kieff, E., and Roizman, B. (1968). Characterization of herpes simplex virus strains differing in their effects on social behavior of infected cells. *J. Gen. Virol.* 2, 357-364.

Feng, Y., Broder, C.C., Kennedy, P.E., and Berger, E.A. (1996). HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272, 872-877.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N., and Minson, T. (1992). Construction and properties of a mutant of herpes simplex virus type 1 with glycoprotein H coding sequences deleted. *J. Virol.* 66, 341-348.

Heller, R.A., and Krönke, M. (1994). Tumor necrosis factor receptor-mediated signaling pathways. *J. Cell Biol.* 126, 5-9.

Highlander, S.L., Sutherland, S.L., Gage, P.J., Johnson, D.C., Levine, M., and Glorioso, J.C. (1987). Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. *J. Virol.* 61, 3356-3364.

Hirt, B. (1967). Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* 26, 365-369.

Hoggan, M.D., and Roizman, B. (1959). The isolation and properties of a variant of herpes simplex producing multinucleated giant cells in monolayer cultures in the presence of antibody. *Am. J. Hyg.* 70, 208-219.

Huang, A.S., and Wagner, R.R. (1964). Penetration of herpes simplex virus into human epidermoid cells. *Proc. Soc. Exp. Biol. Med.* 116, 863-869.

Johnson, R.M., and Spear, P.G. (1989). Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. *J. Virol.* 63, 819-827.

Kaner, R.J., Baird, A., Mansukhani, A., Basilico, C., Summers, B.D., Florkiewicz, R.Z., and Hajjar, D.P. (1990). Fibroblast growth factor receptor is a portal of cellular entry for herpes simplex virus type 1. *Science* 248, 1410-1413.

Klatzmann, D., Champagne, E., Chamaret, S., Gruest, J., Guetard, D., Hercend, T., Gluckman, J.-C., and Montagnier, L. (1984). T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* 312, 767-768.

- Klupp, B.G., Baumeister, J., Karger, A., Visser, N., and Mettenleiter, T.C. (1994). Identification and characterization of a novel structural glycoprotein in pseudorabies virus, gL. *J. Virol.* 68, 3868-3878.
- Ligas, M.W., and Johnson, D.C. (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β -galactosidase sequences binds to but is unable to penetrate into cells. *J. Virol.* 62, 1486-1494.
- Little, S.P., and Schaffer, P.A. (1981). Expression of the syncytial (syn) phenotype in HSV-1, strain KOS: genetic and phenotypic studies of mutants in two syn loci. *Virology* 112, 686-702.
- Maddon, P.J., McDougal, J.S., Clapham, P.R., Dalgliesh, A.G., Jamal, S., Weiss, R.A., and Axel, R. (1988). HIV infection does not require endocytosis of its receptor, CD4. *Cell* 54, 865-874.
- Maness, L.M., Kastin, A.J., Weber, J.T., Banks, W.A., Beckman, B.S., and Zadina, J.E. (1994). The neurotrophins and their receptors: structure, function, and neuropathology. *Neurosci. Biobehav. Rev.* 18, 143-159.
- McGeoch, D.J., Dalrymple, M.A., Davison, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E., and Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* 69, 1531-1574.
- Mirza, D.P., Navarro, D., Paz, P., Lee, P.L., Pereira, L., and Williams, L.T. (1992). The fibroblast growth factor receptor is not required for herpes simplex virus type 1 infection. *J. Virol.* 66, 448-457.
- Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C., and Kieff, E. (1995). The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. *Cell* 80, 389-399.
- Munk, K., and Donner, D. (1963). Cytopathischer Effekt und Plaque-Morphologie verschiedener Herpes-simplex-Virus-Stämme. *Arch. Gesamte Virus-forsch.* 13, 529-540.
- Pelton, B.K., Imrie, R.C., and Denman, A.M. (1977). Susceptibility of human lymphocyte populations to infection by herpes simplex virus. *Immunology* 32, 803-810.
- Pogue-Geile, K.L., and Spear, P.G. (1987). The single base pair substitution responsible for the Syn phenotype of herpes simplex virus type 1, strain MP. *Virology* 157, 67-74.
- Rabizadeh, S., and Bredesen, D.E. (1994). Is p75NGFR involved in developmental neural cell death? *Dev. Neurosci.* 16, 207-211.
- Rinaldo, C.R.J., Richter, B.S., Black, P.H., Callery, R., Chess, L., and Hirsch, M.S. (1978). Replication of herpes simplex virus and cytomegalovirus in human leukocytes. *J. Immunol.* 120, 130-136.
- Roop, C., Hutchinson, L., and Johnson, D.C. (1993). A mutant herpes simplex virus type 1 unable to express glycoprotein L cannot enter cells, and its particles lack glycoprotein H. *J. Virol.* 67, 2285-2297.
- Rothe, M., Wong, S.C., Henzel, W.J., and Goeddel, D.V. (1994). A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell* 78, 681-692.
- Sarmiento, M., Haffey, M.L., and Spear, P.G. (1979). Membrane proteins specified by herpes simplex viruses: role of glycoprotein VP7(B2). *J. Virol.* 29, 1149-1158.
- Shieh, M.-T., and Spear, P.G. (1991). Fibroblast growth factor receptor: does it have a role in the binding of herpes simplex virus? *Science* 253, 208-210.
- Shieh, M.-T., WuDunn, D., Montgomery, R.I., Esko, J.D., and Spear, P.G. (1992). Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J. Cell Biol.* 116, 1273-1281.
- Smith, C.A., Farrah, T., and Goodwin, R.G. (1994). The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 76, 959-962.
- Spear, P.G. (1993). Entry of alphaherpesviruses into cells. *Semin. Virol.* 4, 167-180.
- Subramanian, G., McClain, D.S., Perez, A., and Fuller, A.O. (1994). Swine testis cells contain functional heparan sulfate but are defective in entry of herpes simplex virus. *J. Virol.* 68, 5667-5676.
- Teute, H., Braun, R., Kirchner, H., Becker, H., and Munk, K. (1983). Replication of herpes simplex virus in human T lymphocytes. *Immunology* 20, 32-41.
- Weise, K., Kaerner, H.C., Glorioso, J., and Schröder, C.H. (1987). Replacement of glycoprotein B gene sequences in herpes simplex virus type 1 strain ANG by corresponding sequences of the strain KOS causes changes in plaque morphology and neuropathogenicity. *J. Gen. Virol.* 68, 1909-1919.
- Wickham, T.J., Mathias, P., Cheresh, D.A., and Nemerow, G.R. (1993). Integrins $\alpha_5\beta_1$ and $\alpha_5\beta_3$ promote adenovirus internalization but not virus attachment. *Cell* 73, 309-319.
- Wilson, I.A., Niman, H.L., Houghten, R.A., Cherenon, A.R., Connolly, M.L., and Lerner, R.A. (1984). The structure of an antigenic determinant in a protein. *Cell* 37, 767-778.
- Wittels, M., and Spear, P.G. (1991). Penetration of cells by herpes simplex virus does not require a low pH-dependent endocytic pathway. *Virus Res.* 18, 271-290.

Glycoprotein D of Herpes Simplex Virus (HSV) Binds Directly to HVEM, a Member of the Tumor Necrosis Factor Receptor Superfamily and a Mediator of HSV Entry

J. CHARLES WHITBECK,^{1,2,3*} CHARLINE PENG,¹ HUAN LOU,¹ RULIANG XU,^{1,2,3} SHARON H. WILLIS,^{1,2,3} MANUEL PONCE DE LEON,^{1,2,3} TAO PENG,^{1,2,3} ANTHONY V. NICOLA,^{1,2,3} REBECCA I. MONTGOMERY,⁴ MORGYN S. WARNER,⁴ ATHENA M. SOULIKA,⁵ LYNN A. SPRUCE,⁵ WILLIAM T. MOORE,⁵ JOHN D. LAMBRIS,⁵ PATRICIA G. SPEAR,⁴ GARY H. COHEN,^{1,2} AND ROSELYN J. EISENBERG^{2,3}

School of Dental Medicine,¹ Center for Oral Health Research,² School of Veterinary Medicine,³ and School of Medicine,⁵ University of Pennsylvania, Philadelphia, Pennsylvania 19104, and Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, Illinois 60611⁴

Received 5 March 1997/Accepted 22 April 1997

Glycoprotein D (gD) is a structural component of the herpes simplex virus (HSV) envelope which is essential for virus entry into host cells. Chinese hamster ovary (CHO-K1) cells are one of the few cell types which are nonpermissive for the entry of many HSV strains. However, when these cells are transformed with the gene for the herpesvirus entry mediator (HVEM), the resulting cells, CHO-HVEM12, are permissive for many HSV strains, such as HSV-1(KOS). By virtue of its four cysteine-rich pseudorepeats, HVEM is a member of the tumor necrosis factor receptor superfamily of proteins. Recombinant forms of gD and HVEM, gD-1(306t) and HVEM(200t), respectively, were used to demonstrate a specific physical interaction between these two proteins. This interaction was dependent on native gD conformation but independent of its N-linked oligosaccharides, as expected from previous structure-function studies. Recombinant forms of gD derived from HSV-1(KOS)rid1 and HSV-1(ANG) did not bind to HVEM(200t), explaining the inability of these viruses to infect CHO-HVEM12 cells. A variant gD protein, gD-1(Δ 290-299t), showed enhanced binding to HVEM(200t) relative to the binding of gD-1(306t). Competition studies showed that gD-1(Δ 290-299t) and gD-1(306t) bound to the same region of HVEM(200t), suggesting that the differences in binding to HVEM are due to differences in affinity. These differences were also reflected in the ability of gD-1(Δ 290-299t) but not gD-1(306t) to block HSV type 1 infection of CHO-HVEM12 cells. By gel filtration chromatography, the complex between gD-1(Δ 290-299t) and HVEM(200t) had a molecular mass of 113 kDa and a molar ratio of 1:2. We conclude that HVEM interacts directly with gD, suggesting that HVEM is a receptor for virion gD and that the interaction between these proteins is a step in HSV entry into HVEM-expressing cells.

The envelope of herpes simplex virus (HSV) is complex, as it contains at least 10 virus-encoded glycoproteins (53). However, only a subset of these mediate virus entry in cell culture. The initial interaction of HSV with cell surface heparan sulfate proteoglycans is mediated by glycoprotein C (gC) and/or gB (19, 20, 62). This is presumably followed by interaction of one or more of the viral glycoproteins with cellular receptors (6, 25, 26, 29). Then gD, gB, and the complex of gH and gL act individually or in combination to trigger pH-independent fusion of the viral envelope with the host cell plasma membrane (53).

Several lines of evidence have implicated gD as an HSV receptor-binding protein. For example, UV-inactivated wild-type HSV virions, but not UV-inactivated virions lacking gD, are able to block infection by HSV (26, 29). Second, wild-type strains of HSV do not infect cells expressing gD (5, 27). This gD-mediated interference occurs at the level of penetration and is dependent on the structure of gD in the infecting virus (6, 11, 12, 27, 44). Lastly, cells incubated in the presence of soluble, truncated gD (gDt) are resistant to infection (17, 25, 39, 55). A unifying explanation for these observations is that inhibition is primarily due to binding of nonvirion gD to cel-

lular receptor(s) which prevents them from binding to gD in the virus.

gD is a typical type I integral membrane glycoprotein. In its ectodomain of 319 amino acids, gD has three sites for the addition of N-linked oligosaccharides (N-CHO) and six cysteine residues arranged into three disulfide bonds (31). gD function is dependent on its native conformation but is independent of the three N-CHO (9, 15, 51, 52, 54). Mutagenesis coupled with complementation analysis identified four separate regions of gD that are important for virus entry (7, 37). Wild-type and mutant forms of gDt were cloned into a baculovirus expression system, and the abilities of these proteins to block HSV infection were studied. The ability of gDt to inhibit HSV infection depends on its native conformation (39) but is independent of the presence of the three N-CHO (60). Some mutations ablated the ability of gDt to block infection, whereas others, such as gD-1(Δ 290-299t), markedly enhanced blocking activity. We speculated that changes in gDt structure altered its ability to interact with cellular protein(s), i.e., receptor(s) (38, 39). This hypothesis could best be verified by identifying the cellular receptor(s) which interacts with gD to mediate HSV entry.

Recently, expression cloning was used to isolate and identify a HeLa cell gene product which upon expression in normally nonpermissive Chinese hamster ovary (CHO) cells allows for

* Corresponding author. Phone: (215) 898-6553. Fax: (215) 898-8385.

entry of many HSV strains (35). This gene product, the herpesvirus entry mediator (HVEM), is a 230-amino-acid type I integral membrane protein. Because it contains a motif of four cysteine-rich pseudorepeat sequences, it is considered a new member of the tumor necrosis factor receptor (TNFR) superfamily (1, 50). A soluble form of HVEM (consisting of the ectodomain of HVEM fused in frame to the C_{H2} and C_{H3} domains of rabbit immunoglobulin G [IgG] heavy chain), HVEM:Fc, blocks HSV type 1 (HSV-1) infection of CHO cells stably transformed to express HVEM on the cell surface (CHO-HVEM12 cells). Moreover, polyclonal antibodies to HVEM:Fc block HSV-1 infection of CHO-HVEM12 cells. Three HSV-1 strains with changes in the gD sequence infected CHO-HVEM12 cells with markedly reduced efficiencies, suggesting that HVEM interacts directly with gD.

In this study, our goal was to test the hypothesis that gD can interact directly with HVEM. The approach was to use purified truncated forms of both HVEM and viral glycoproteins in direct binding assays. Among the five herpesvirus glycoproteins involved in entry, only gD was able to bind to HVEM. The binding was dependent on the native conformation of gD but independent of its N-CHO. These and other studies presented here show that HVEM meets the criteria for a receptor which interacts with gD and leads to HSV entry.

MATERIALS AND METHODS

Cells and virus. African green monkey (Vero) cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 5% fetal bovine serum (FBS). Vero-gL cells, stably transfected with a plasmid expressing HSV-1 gL (35, 40), were grown in DMEM-10% FBS with G418 (400 µg/ml). CHO-HVEM12 cells (35) were grown in Ham's F-12 medium supplemented with 10% FBS and 200 µg of G418 per ml. Sf9 (*Spodoptera frugiperda*) cells (GIBCO BRL) were grown in Sf900II medium (GIBCO BRL). KOS-gL86 is a mutant KOS virus in which the *Escherichia coli lacZ* gene under the control of the cytomegalovirus immediate-early promoter replaces part of the gL open reading frame (ORF) (35, 40). This virus was propagated on Vero-gL cells.

Construction of a baculovirus recombinant expressing a truncated and secreted form of HVEM. The strategy used was the same as that employed in the construction of a baculovirus recombinant expressing gD-1(306t) (49). Plasmid pBEC10 contains the entire coding sequence for the HVEM ORF (35). A 486-bp fragment of HVEM, corresponding to amino acids 39 to 200 (where amino acid 39 is the first amino acid after the predicted signal peptide sequence), was amplified by PCR. The amino-terminal primer, 5'-GCGAGATCTGCCATCAT GCAAGGAGGACGAGTA-3', hybridized to the noncoding strand of HVEM and added a *Bgl*II site (bold letters) just upstream from the codon for amino acid 39. The carboxy-terminal primer, 5'-GCGTGATCAGTGGTGGTGGTGGTGG TGGGAGCTGCTGGTTCAGCT-3', added five histidine codons after the histidine at amino acid 200 of HVEM, a stop codon, and a *Bcl*I site (bold letters). The histidine codons were added to provide a binding site for nickel-nitriloacetic acid-agarose resin (Qiagen) for purification (see below). The PCR-amplified product was digested with *Bgl*II and *Bcl*I and ligated with DNA from plasmid pVT-Bac (56) which had been digested with *Bam*HI. The mellitin signal sequence, coded for by pVT-Bac, replaced the HVEM signal. An extra aspartic acid codon was added to the N terminus of HVEM as a result of cloning (Fig. 1A). The ligated plasmid was used to transform XL2-Blue (Stratagene) competent *E. coli* cells. The resulting plasmid, pCW275, was recombined into baculovirus (*Autographa californica* nuclear polyhedrosis virus) by cotransfection with Baculogold DNA (Pharmingen) (49). Plaques were picked and amplified. Culture supernatants were screened for HVEM expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting). Blots were probed with rabbit anti-HVEM peptide serum R133 (see below) (Fig. 1A). Baculovirus recombinants expressing HVEM were subjected to two additional rounds of plaque purification (49). The recombinant baculovirus was named bac-HVEM(200t). The recombinant protein was designated HVEM(200t) (Fig. 1A).

Purification of HVEM(200t). Sf9 cells grown in 3-liter suspension cultures (61) were infected with bac-HVEM(200t) at a multiplicity of infection of 4. At 48 h postinfection, the supernatant was clarified by centrifugation (1,500 × g for 30 min at 4°C) and then by filtration (0.22-µm-pore-size filter). The supernatant was concentrated and exchanged into 600 ml of phosphate-buffered saline (PBS) by tangential flow filtration (10-kDa molecular mass cutoff membrane; Millipore). The protein solution was mixed with 2.5 ml of nickel-nitriloacetic acid resin (Qiagen; pre-equilibrated with PBS) and incubated overnight at 4°C on a rotary shaker. The resin was pelleted (100 × g for 10 min at 4°C), resuspended in PBS, transferred to a column, and washed first with PBS and then with stepwise

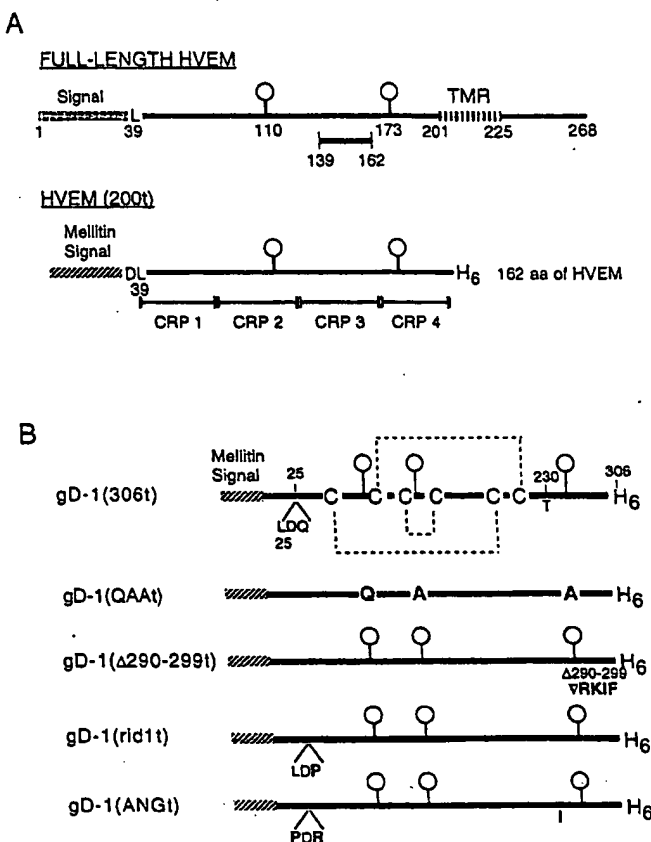


FIG. 1. Schematic representations of gD and HVEM proteins. (A) Diagrams of the full-length HVEM from HeLa cells (35) and the recombinant baculovirus protein HVEM(200t). Leucine 39 is the first amino acid residue of the mature protein (after removal of the predicted signal sequence). In HVEM(200t), the natural signal was replaced by the honeybee mellitin signal encoded by the insertion vector pVT-Bac (56). Cloning of the HVEM ectodomain ORF into pVT-Bac also added an aspartic acid residue to the N-terminus. HVEM(200t) was truncated at histidine 200 prior to the predicted transmembrane region (TMR) and five additional histidine residues were added to the C terminus. Consensus sites for the addition of N-CHO are indicated by balloons. The 24-amino-acid (aa) peptide (aa residues 139 to 162) used to prepare R133 antiserum is shown below the full-length form of HVEM. The boundaries of the four cysteine-rich pseudorepeat (CRP) elements present in the HVEM ectodomain are shown below HVEM(200t). (B) Forms of HSV-1 gD produced by recombinant baculovirus-infected cells (38, 39, 49). Each form is truncated prior to the TMR and contains a six-histidine tag at the carboxy terminus. gD-1(306t) (Patton strain) has the same sequence as gD from strain KOS through amino acid 306. Not shown is gD-2(306t) from HSV-2 strain 333 (39). N-CHO sites are indicated by balloons. Cysteine residues, along with the disulfide bonding pattern determined for full length gD-1 (31), are also shown. gD-1(QAA) has three amino acid changes relative to gD-1(306t), N94Q, S123A, and T264A, resulting in a molecule lacking N-CHO (49). gD-1(Δ290-299t) contains a combined deletion and linker-insertion mutation where amino acid residues 290 through 299 of gD-1(306t) (IPPNWHIPSI) are replaced by the amino acid residues RKIF (7, 39). gD-1(Δ290-299t) has a single amino acid change (Q27P), and gD-1(ANGt) has three amino acid changes (L25P, Q27R, and T230I) relative to gD-1(306t) (38).

increasing concentrations of imidazole (0.01 to 0.25 M) in 0.02 M phosphate buffer (pH 7.5) containing 0.5 M NaCl. The 0.25 M eluate, containing most of the HVEM(200t), was dialyzed against PBS and concentrated (10-kDa molecular mass centrifugal membrane; Millipore). The yield of purified HVEM(200t) was 2 to 3 mg/liter of supernatant.

Production and purification of soluble forms of HSV glycoproteins. The production and purification of gD-1(306t), gD-2(306t), gD-1(QAA), gD-1(Δ290-299t), gD-1(Δ290-299t), gD-1(ANGt), and gC-1(Δ57t) (Fig. 1B) from recombinant baculovirus-infected cells have been described elsewhere (38, 39, 49, 55). HSV-1 gH truncated at amino acid 792 and complexed to full-length HSV-1 gL was produced by mouse L cells stably transformed with plasmids pCMV3gH(792) and pCMV3gL-1 (14). This cell line, HL-7, was kindly provided by Gary Dubin.

The properties of these cells and gHt-gL purification protocols are described in detail elsewhere (41). gB-1s was produced in 293 (human kidney) cells stably transformed with plasmid pRP-RSV-gBs (32). These cells, which were kindly provided by R. Manservigi, were maintained in DMEM supplemented with 10% FBS and 3 μ g of methotrexate per ml. To obtain secreted protein, cells were grown for 24 h in serum-free medium lacking methotrexate and the supernatant was applied to a column of anti-gB-1 monoclonal antibody (MAb) A22-Sepharose. The column was washed with 0.01 M Tris (pH 7.5) containing 0.15 M NaCl and then eluted with 3 M KSCN. The eluate was dialyzed against PBS and concentrated (PM10 membrane; Amicon). Soluble purified CD4 was kindly provided by Robert Doms, and sTva, a recombinant baculovirus-produced form of Tva, the receptor for Rous sarcoma virus (18), was kindly provided by Paul Bates.

Antibodies. Monospecific antiserum (R133) to HVEM was generated by immunizing a rabbit with peptide RRYATSSPGQRVQKGGTESQDTLC (Fig. 1A) coupled to keyhole limpet hemocyanin as previously described (8). R133 specifically recognizes the synthetic peptide used for immunization. The production of antiserum to HVEM:Fc was previously described (35). R7 antiserum was raised against gD-2 isolated from virus-infected cells (23). R46 antiserum was raised against gC isolated from HSV-1-infected cells (16). R69 antiserum was prepared against reduced and alkylated gB isolated from HSV-1-infected cells (16). MAbs 37S (48) and 8H4 (14) were used to detect gH and gL, respectively. MAb A22.1, used for gB purification, was kindly supplied by Becton Dickinson.

SDS-PAGE analysis. Purified glycoproteins were separated by SDS-PAGE under reducing conditions in precast Tris-glycine gels (Novex). After SDS-PAGE, separated proteins were either silver stained (Pharmacia) or transferred to nitrocellulose and reacted with the appropriate antiserum. Blots were blocked with PBS containing 5% milk and 0.2% Tween 20 and incubated with secondary antibody (goat anti-mouse or goat-anti rabbit) coupled to horseradish peroxidase in PBS containing 5% milk and 0.2% Tween 20. Blots were washed with 0.2% Tween 20 in PBS, and bands were visualized by exposure to X-ray film after the addition of chemiluminescent substrate (ECL; Amersham). Purified glycoproteins were digested with glycosidases as previously described (45, 49).

Blocking of HSV-1 entry into CHO-HVEM12 and Vero cells by soluble gD or soluble HVEM. Vero and CHO-HVEM12 cells were plated on 96-well dishes and incubated overnight. For experiments with soluble gD, cells were chilled to 4°C for 10 min and the medium was replaced with DMEM-5% FBS containing various concentrations of gD-1(306t) or gD-1(Δ 290-299t). The plates were rocked for 90 min at 4°C, at which time KOS-gL86 (5×10^5 PFU/well) was added. The plates were rocked for an additional 90 min at 4°C, and then DMEM-5% FBS containing the appropriate soluble gD was added to maintain the initial concentration of soluble gD during the subsequent 6 h of incubation at 37°C. The experiments with soluble HVEM were done as previously described (35). Briefly, KOS-gL86 was mixed with various concentrations of HVEM(200t), HVEM:Fc (35), or rabbit IgG as a control and then incubated for 30 min at 37°C before addition to CHO-HVEM12 cells on 96-well plates. After 2 h of incubation at 37°C, cells were treated with 0.1 M citrate buffer (pH 3.0) to inactivate extracellular virus. Cells were washed and incubated in PBS containing glucose and 1% calf serum for 4 h at 37°C. In both types of assays, cells were washed with PBS at 6 h postinfection, substrate (o-nitrophenyl- β -D-glucopyranoside in PBS containing 0.5% Nonidet P-40) was added to each well, and β -galactosidase activity was measured at various time points with a Spectromax 250 enzyme-linked immunosorbent assay (ELISA) reader. The results were plotted as percentages of controls in which no soluble gD or HVEM was present.

ELISA. Soluble receptor proteins [HVEM(200t), CD4, and sTva] in PBS were bound to wells of microtiter plates for 3 h at room temperature (RT). Plates were washed with 0.1% Tween 20 in PBS (PBS-Tween) and incubated in 5% nonfat milk-0.2% Tween 20 in PBS (blocking solution) for 30 min at RT. Plates were washed with PBS-Tween and incubated with gD (or other HSV-1 envelope glycoproteins) at various concentrations in blocking solution for 16 h at 4°C. Plates were washed with PBS-Tween and incubated for 30 min at RT with the appropriate antiserum diluted in blocking solution. Plates were washed three times with PBS-Tween and incubated with horseradish peroxidase-conjugated secondary antibody diluted in blocking solution. Plates were washed once with PBS-Tween and then with 20 mM citrate buffer (pH 4.5). A substrate of 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (Moss, Inc.) in citrate buffer (pH 4.5) was added, and the A_{405} was read with a microtiter plate reader (Bio-Tek).

Competition binding assay with 125 I-labeled gD-1(Δ 290-299t). HVEM(200t) in PBS at a concentration of 200 nM was used to coat modular 96-well microtiter plates for 3 h at RT. Plates were washed and blocked as described above for ELISA. Serial threefold dilutions of unlabeled competitor proteins in blocking solution were added to duplicate wells of the plate, and 125 I-labeled gD-1(Δ 290-299t), iodinated as previously described (36), was added to a final concentration of 14 nM. Plates were incubated for 16 h at 4°C and washed with PBS-Tween. Wells were separated and counted in a gamma counter (Wallac). At each concentration of competitor, the percentage of 125 I-labeled gD-1(Δ 290-299t) bound was calculated as follows: [counts per minute of 125 I-labeled gD-1(Δ 290-299t) bound in the presence of competitor/counts per minute of 125 I-labeled gD-1(Δ 290-299t) without competitor] \times 100.

Mass spectrometry, gel filtration, and N-terminal sequencing. Matrix-assisted laser desorption-ionization mass spectrometry (21) was performed with samples of HVEM(200t) dissolved in 50% acetonitrile containing 1% trifluoroacetic acid

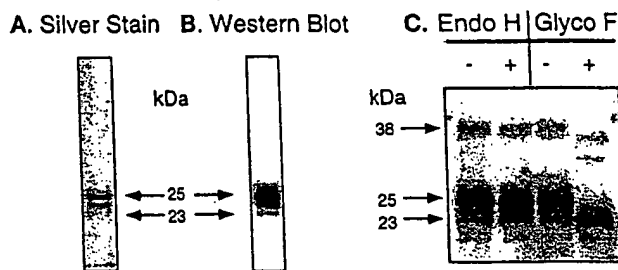


FIG. 2. SDS-PAGE analysis of purified HVEM(200t). Purified HVEM(200t) was electrophoresed through a 12% Tris-glycine-polyacrylamide gel under reducing conditions and visualized by silver staining (A) or Western blotting with R133 antiserum (B). The sizes of stained protein bands were calculated from the migration of molecular mass standards. (C) Purified HVEM(200t) was incubated under identical conditions without (–) or with (+) endo H or glyco F. Mock- and enzyme-digested samples were electrophoresed under reducing conditions through a 12% Tris-glycine-polyacrylamide gel, Western blotted, and probed with R133 antiserum.

and diluted with 2-(4-hydroxyphenylazo)benzoic acid (Aldrich) as previously described (45). For gel filtration, proteins were diluted with PBS and were applied to a calibrated Superdex 200 column (HR 10/30; Pharmacia). Fractions were collected and analyzed for HVEM(200t) and gD-1(Δ 290-299t) by SDS-PAGE and subsequent immunoblotting with R133 antiserum to visualize HVEM and R7 antiserum to visualize gD. Samples of HVEM(200t) and gD-1(Δ 290-299t), as well as the complex of the two proteins isolated by size exclusion chromatography, were sequenced by adsorptive protocols with a Perkin-Elmer/Applied Biosystems 473A microsequencer (45).

RESULTS

Characterization of baculovirus-produced HVEM(200t). Previously we constructed a plasmid which upon transfection into mammalian cells produced the ectodomain of HVEM fused to the Fc region of rabbit IgG (HVEM:Fc) (35). For the production of large quantities of HVEM, we expressed the ectodomain with a C-terminal, six-histidine tag, HVEM(200t), in the baculovirus system (Fig. 1A). HSV glycoproteins expressed in insect cells from baculovirus recombinants have properties similar to those of proteins produced in mammalian cells (38, 39, 45, 49, 55).

HVEM(200t) was purified by nickel-agarose chromatography and analyzed by SDS-PAGE (Fig. 2A and B). The protein was present primarily in three closely migrating silver-stained bands (Fig. 2A), ranging from 23 to 25 kDa. A faint band which migrated to 38 kDa was sometimes seen (Fig. 2C). On Western blots, the same bands also reacted with both R133 and anti-HVEM:Fc (35) (data not shown).

The deduced HVEM amino acid sequence includes two potential sites for the addition of N-CHO, and HVEM:Fc was shown to be N glycosylated (35). Purified HVEM(200t) was digested with endoglycosidase H (endo H) and glycopeptidase F (glyco F) to verify that it also contained such posttranslational N-CHO modifications (Fig. 2C). Treatment with endo H had no effect, whereas treatment with glyco F increased the mobility of HVEM(200t). The simplest interpretation is that the closely migrating bands in the control lane represent HVEM(200t) with two, one, or no mature (Golgi-modified) forms of N-CHO. The mobility of the 38-kDa band was also altered by glyco F, suggesting that it was a dimeric form of HVEM(200t) which survived the denaturation conditions.

Because HVEM(200t) exhibited heterogeneity by SDS-PAGE, we examined it by mass spectrometry (Fig. 3A). HVEM(200t) was detected as a major peak of 20,020 Da and two minor peaks, with each one about 700 Da smaller. The minor peaks may represent HVEM(200t) with one or no N-

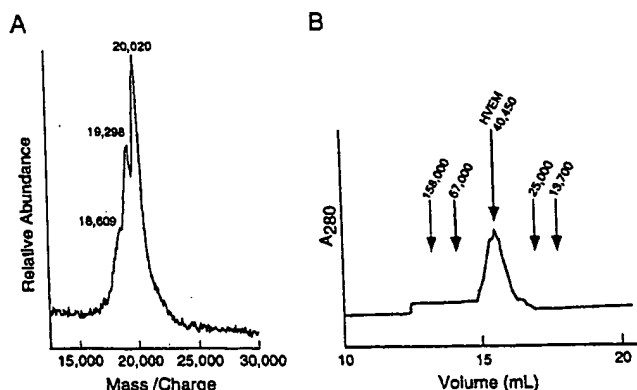


FIG. 3. Analysis of the molecular size of purified HVEM(200t). (A) Purified HVEM(200t) was dissolved in 50% acetonitrile containing 1% trifluoroacetic acid, diluted with 2-(4-hydroxyphenylazo)benzoic acid, and analyzed by mass spectrometry. The calculated masses of the three peaks of singly charged species are indicated. (B) HVEM(200t) (35 μ M in PBS) was loaded onto a Superdex 200 gel filtration column and eluted with PBS. The eluate was monitored for protein by A_{280} . The major A_{280} peak had a calculated molecular mass of 40,450 Da, based on the peak positions of molecular mass standards (indicated by arrows).

CHO (45). No covalently linked dimeric species were detected. N-terminal sequencing of purified HVEM(200t) confirmed the expected amino acid sequence through 10 residues (data not shown). No other sequences were present, suggesting that the size heterogeneity of HVEM(200t) is due primarily to heterogeneity of N-CHO.

By gel filtration (Fig. 3B), HVEM(200t) eluted with a calculated mass of 40 kDa, suggesting that it is a dimer in solution. SDS-PAGE and immunoblot analyses of the peak fractions from the gel filtration column indicated that the 40-kDa form contained all three glycosylation isoforms (data not shown).

It was previously shown that HVEM:Fc blocks HSV-1(KOS) infection of CHO-HVEM12 cells (35). To determine whether HVEM(200t) had similar biological activity, HVEM(200t), HVEM:Fc, and rabbit IgG were tested for the ability to block HSV-1(KOS) entry into CHO-HVEM12 cells (Fig. 4). The results showed that the two recombinant forms of HVEM were equally effective in blocking HSV infection. In both cases, the concentration necessary to inhibit infection by 50% was 1 μ M. In this experiment, the rabbit IgG control had some inhibitory effect when it was present at high concentrations, possibly due to interaction with gE and gI (13). The results suggested that both HVEM(200t) and HVEM:Fc interacted with at least one virion component essential for virus entry.

Evidence that gD binds directly to HVEM. Having demonstrated that HVEM(200t) had biological activity, we next used ELISA to examine the *in vitro* interaction between gD-1(306t) and HVEM(200t) (Fig. 5). Microtiter plates were coated with various concentrations of HVEM(200t), and then the plates were incubated with various concentrations of gD-1(306t) (49), ranging from 1 nM to 1 μ M. Bound gD was detected with anti-gD serum R7. Increasing amounts of gD-1(306t) bound as the concentration of HVEM(200t) increased, up to a concentration of 200 nM (Fig. 5A). No binding was detected in control wells containing no HVEM(200t). The experiment was repeated at 200 nM HVEM(200t) and with a greater range of gD-1(306t) concentrations (up to 20 μ M) to be certain that the reaction was saturable (Fig. 5B). Saturation occurred at 5 μ M gD-1(306t). In subsequent experiments, plates were coated with 200 nM HVEM(200t).

Is the reaction specific for HVEM and gD? To be certain we were not detecting nonspecific binding to a glycoprotein receptor, we carried out two experiments (Fig. 6). First, we asked whether secreted forms of other HSV glycoproteins involved in entry (i.e., gB, gC, and gH-gL) could bind to HVEM(200t). Each of these proteins was purified by immunoaffinity chromatography, and each reacted as expected with several different MABs (14, 33, 39, 42, 55) (data not shown). Various concentrations of gD-1(306t), gD-2(306t) (39), gC-1(457t) (55), gB-1s (33), and gHt-gL (42) were incubated on ELISA plates coated with HVEM(200t), and binding was detected with antibody to each glycoprotein. gD-1(306t) and gD-2(306t) bound similarly to HVEM(200t) (Fig. 6A); however, gC-1(457t), gB-1s, and gHt-gL failed to bind, indicating the specificity of HVEM binding for gD. The fact that gD-1(306t) and gD-2(306t) bound similarly to HVEM(200t) is consistent with the finding that the entry of both HSV-1 and HSV-2 strains into CHO cells was enhanced by the expression of HVEM (35). Both forms of gD can also block HSV infection of Vero cells (39), although the mediator(s) of entry into these cells has not yet been identified.

As a second test of specificity, we examined the binding of gD-1(306t) to soluble forms of extraneous viral receptors. gD-1(306t) bound to ELISA plates coated with HVEM(200t) but not to plates coated with CD4 (the HIV-1 receptor) or sTva (a soluble form of the Rous sarcoma virus receptor) (18) (Fig. 6B). In separate experiments, neither gD-1(306t) nor gD-1(Δ 290-299t) bound to purified soluble mannose-6-phosphate receptor (4) (data not shown). Thus, the observed binding was specific both for gD and for HVEM.

Binding of HVEM to gD depends on gD conformation but not on N-CHO on gD. The antigenic structure of gD and its function in infection are highly dependent on its native conformation and the maintenance of its three disulfide bonds (15, 31). Denaturation of gD destroys its ability to interact with

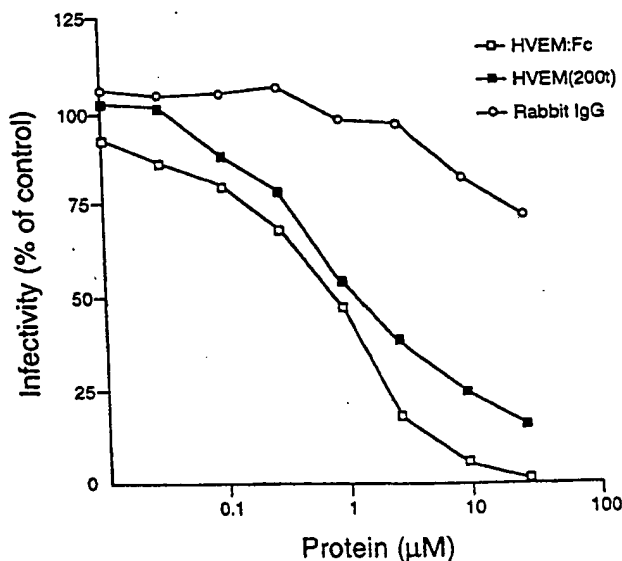


FIG. 4. Effects of soluble HVEM on HSV-1 infection CHO-HVEM12 cells. Various concentrations of HVEM(200t), HVEM:Fc, and rabbit IgG were incubated with KOS-gL86 for 30 min at 37°C prior to inoculation of cells on 96-well plates. After 2 h of incubation at 37°C, cells were treated with low-pH buffer to inactivate extracellular virus, washed, and then incubated for an additional 4 h. Cells were lysed for quantitation of β -galactosidase activity, which was proportional to the number of infected cells and was expressed as a percentage of the activity detected in the absence of soluble HVEM.

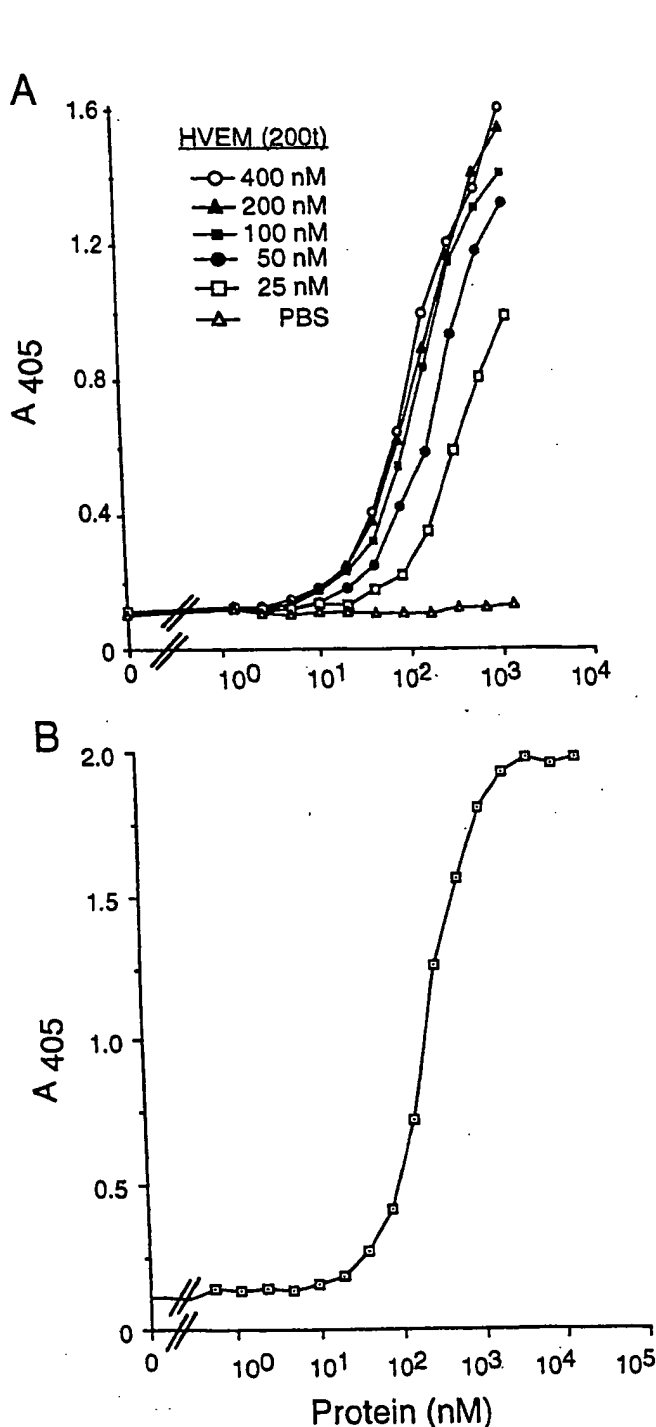


FIG. 5. Binding of gD-1(306t) to HVEM(200t). (A) Various concentrations of HVEM(200t) were bound to wells of an ELISA plate in PBS. Various concentrations of gD-1(306t) were added to the bound HVEM(200t) for 16 h at 4°C. Bound gD was detected with anti-gD serum R7, followed by peroxidase-conjugated secondary antibody and substrate. The data are the averages of duplicate wells. The experiment was repeated twice with similar results. (B) HVEM(200t) at a concentration of 200 nM in PBS was bound to an ELISA plate and then incubated with various concentrations of gD-1(306t) for 16 h at 4°C. Bound gD was detected with R7 antiserum, followed by peroxidase-conjugated secondary antibody and substrate. The data are the averages of duplicate wells.

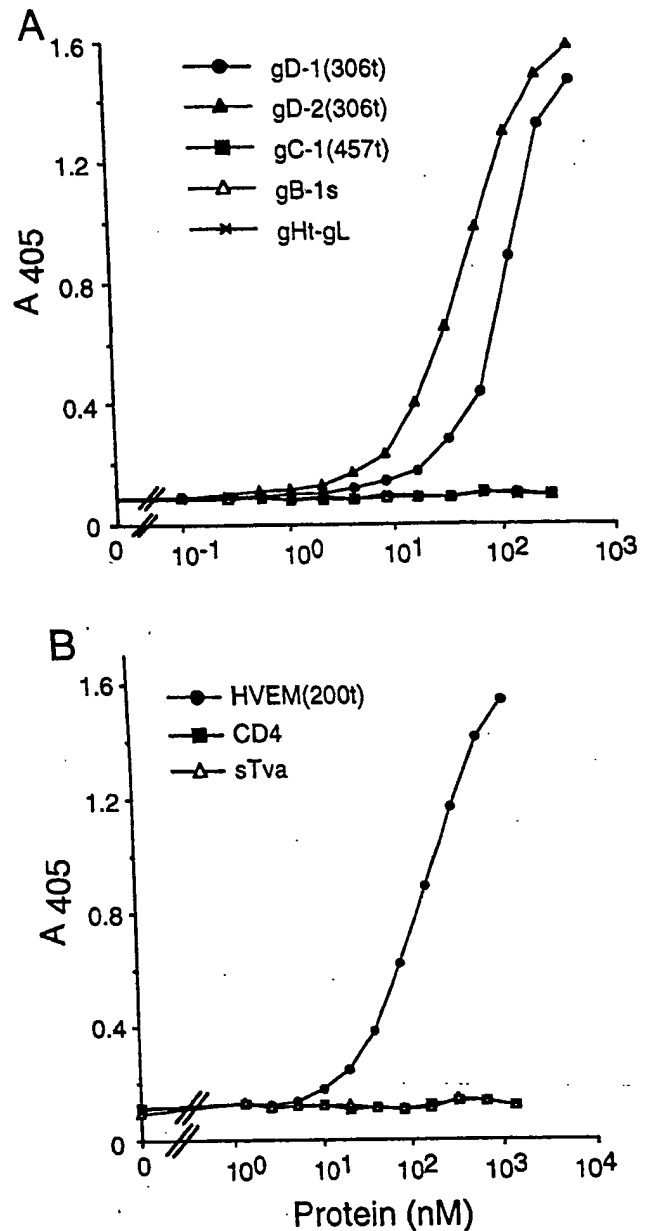


FIG. 6. Specificity of the gD-HVEM interaction. (A) HVEM(200t) at 200 nM in PBS was adsorbed to ELISA plates and incubated with various concentrations of gD-1(306t), gD-2(306t), gC-1(457t), gB-1s, and gHt-gL for 16 h at 4°C. Bound proteins were detected with antiserum R7 (for gD), R47 (for gC), R69 (for gB), or a mixture of MAbs 37S and 8H4 (for gH and gL, respectively), followed by peroxidase-conjugated secondary antibody and substrate. The data are averages of duplicate wells. The experiment was repeated twice with similar results. (B) Wells of an ELISA plate were coated with 200 nM HVEM(200t), CD4, or sTva in PBS and then incubated with various concentrations of gD-1(306t) for 16 h at 4°C. Bound gD was detected with antiserum R7, followed by peroxidase-conjugated secondary antibody and substrate. The data are the averages of triplicate wells. The experiment was repeated twice with similar results.

conformation-dependent MAbs and results in the loss of its biological activity (39). In contrast, gD function is retained when the three sites for N-CHO addition are mutated (51, 52). A variant form of gD, gD-1(QAAt), which lacks signals for N-CHO (Fig. 1B) (49) is able to block infection of Vero cells as effectively as wild-type gD does (46, 60). These properties

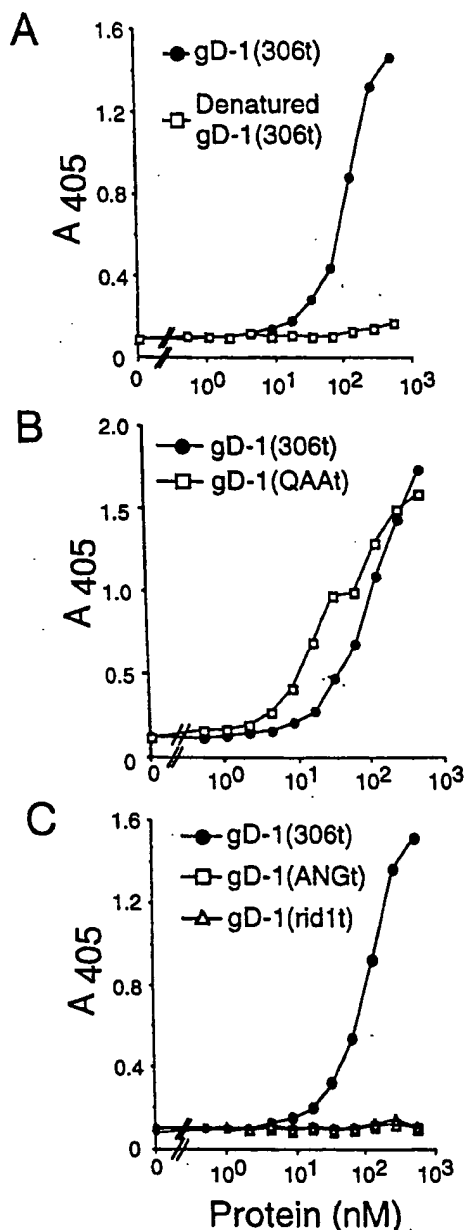


FIG. 7. Binding of denatured and mutant forms of gD to HVEM(200t). ELISA plates were coated with 200 nM HVEM(200t) in PBS, blocked, and incubated with various concentrations of gD. Bound gD was detected with antiserum R7, followed by peroxidase-conjugated secondary antibody and substrate. The data are the averages of duplicate wells, and each experiment was repeated twice with similar results. (A) The binding of gD-1(306t) was compared with that of reduced and alkylated gD-1(306t). gD was reduced and alkylated by previously described methods (45), desalted, and exchanged into PBS on a Pierce polyacrylamide 6000 desalting column. (B) The binding of gD-1(306t) was compared with that of gD-1(QAAt). (C) The binding of gD-1(306t) was compared with those of gD-1(ANGt) and gD-1(rid1t).

predicted that if binding of gD to HVEM is relevant to gD function in vivo, then in vitro binding of gD to HVEM should be dependent on disulfide bonds but independent of N-CHO on gD. gD-1(306t) was reduced and alkylated to ensure that there would be no refolding during the incubation period with HVEM, and this treatment ablated its binding to HVEM(200t) (Fig. 7A). In contrast, gD-1(QAAt) bound as well as gD-1

(306t) did to HVEM(200t) (Fig. 7B). Thus, HVEM has requisite properties of a gD-binding viral receptor.

HVEM does not bind to gD proteins containing the rid1 and ANG mutations. The expression of HVEM by CHO-HVEM12 cells enhances the entry of several wild-type strains of HSV-1 and HSV-2 (35). However, three strains of HSV-1 known to carry mutations in the gD gene did not exhibit enhanced entry into CHO-HVEM12 cells, although they are fully infectious for other cell types such as Vero cells. These viruses were HSV-1(KOS)rid1 (KOS-rid1), KOS-rid2, and HSV-1(ANG). KOS-rid1 and KOS-rid2 were selected for resistance to gD-mediated interference, and both exhibited a change in amino acid 27 of gD (Q27P and Q27R, respectively) (11). HSV-1(ANG) gD has the same substitution at amino acid 27 as KOS-rid2 gD does as well as changes at amino acids 25 (L25P) and 230 (T230I) (24). There are also several changes within the cytoplasmic tail of HSV-1(ANG) relative to HSV-1(KOS). The failure of these viruses to infect CHO-HVEM12 cells could be due to the inability of gD from these strains to bind to HVEM. Therefore, we tested the abilities of truncated versions of these proteins, gD-1(rid1t) and gD-1(ANGt) (Fig. 1B), to bind to HVEM(200t). The biological and structural properties of these proteins have been described previously (38).

Neither gD-1(rid1t) nor gD-1(ANGt) bound to HVEM(200t) in an ELISA (Fig. 7C), thus accounting for the inability of viruses containing these gDs to infect CHO-HVEM12 cells. It is worth noting that both of these proteins are able to block HSV infection of Vero cells (38). The results support the concept that the entry of KOS-rid1 and HSV-1(ANG) into permissive cells involves cellular proteins other than (or in addition to) HVEM (35, 57).

gD-1(Δ 290-299t) shows enhanced binding to HVEM(200t). Baculovirus-produced gDt blocks HSV-1 and HSV-2 infections of Vero and other mammalian cells (38, 39, 55). Among several gDt variants, gD-1(Δ 290-299t) (Fig. 1B) exhibited unexpectedly enhanced inhibitory activity. This observation predicted that gD-1(Δ 290-299t) might bind to HVEM(200t) better than gD-1(306t) did, and we tested this by ELISA (Fig. 8A). Comparable binding to HVEM(200t) occurred at concentrations of gD-1(Δ 290-299t) that were considerably lower than those of gD-1(306t).

The differences in the binding of the two forms of gDt to HVEM(200t) could be either simply quantitative [i.e., gD-1(Δ 290-299t) has a higher affinity for HVEM] or qualitative as well (i.e., the two proteins could interact with different regions of HVEM). To distinguish between these possibilities, we carried out binding competition studies with 125 I-labeled gD-1(Δ 290-299t) (Fig. 8B). A fixed amount of iodinated gDt [the concentration necessary to give half-maximal binding to HVEM(200t)] was mixed with increasing concentrations of unlabeled gD-1(Δ 290-299t) or gD-1(306t) and then added to HVEM(200t). Both proteins competed with the iodinated probe for binding to HVEM(200t), indicating that both forms of gD bound to the same site. Approximately 50-fold-more gD-1(306t) was needed to achieve the same level of blocking as that of a given concentration of gD-1(Δ 290-299t), suggesting that the enhanced binding of gD-1(Δ 290-299t) is due to a higher affinity of interaction for HVEM(200t).

The results of the binding experiment (Fig. 8A) suggest a larger difference in the affinities of the two proteins for HVEM than do the results of the competition experiment (Fig. 8B). Because the signal in Fig. 8A depends on antibodies for the detection of bound gDt, it is possible that some of this apparent enhanced binding is due to increased binding of antibody to the variant protein. However, in separate experiments, we did not see substantial differences in the binding of R7 to gD-1

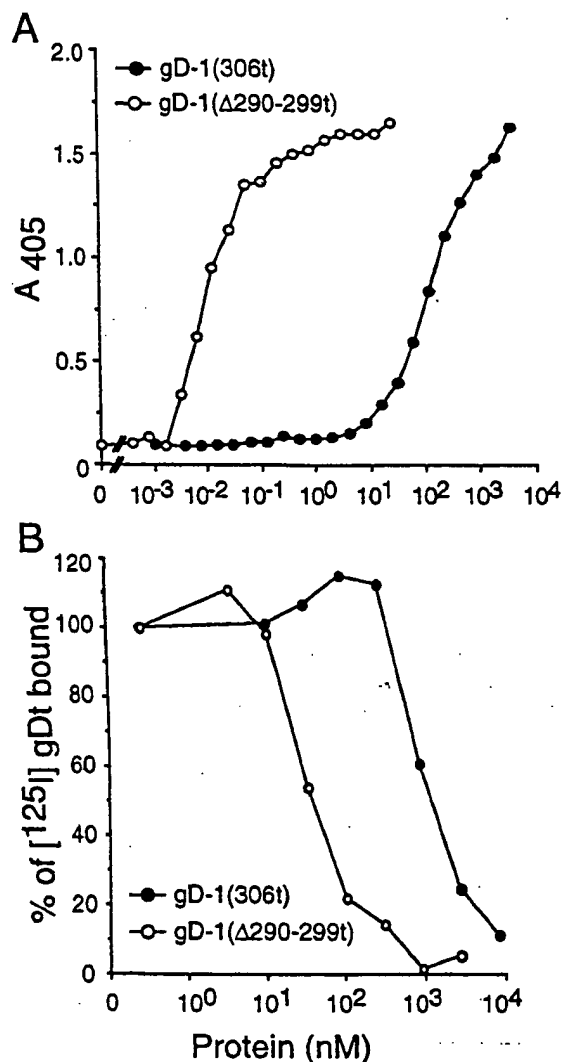


FIG. 8. Binding of gD-1(Δ290-299t) to HVEM(200t). (A) An ELISA plate was coated with 200 nM HVEM(200t) in PBS, blocked, and incubated with various concentrations of gD-1(306t) and gD-1(Δ290-299t). Bound gD was detected with antiserum R7, followed by peroxidase-conjugated secondary antibody and substrate. The data are the averages of duplicate wells, and the experiment was repeated several times with similar results. (B) An ELISA plate was coated with 200 nM HVEM(200t) in PBS, blocked, and incubated with 14 nM ¹²⁵I-labeled gD-1(Δ290-299t) in the presence of various concentrations of unlabeled gD-1(306t) and gD-1(Δ290-299t). Individual wells were separated and counted in a gamma counter. The data are the averages of duplicate wells at each concentration of competitor. The percentage of ¹²⁵I-labeled gD-1(Δ290-299t) bound was calculated as follows: [counts per minute of ¹²⁵I-labeled gD-1(Δ290-299t) bound in the presence of competitor/counts per minute of ¹²⁵I-labeled gD-1(Δ290-299t) without competitor] × 100.

(Δ290-299t) versus that to gD-1(306t) (data not shown). Alternatively, alterations in gD structure resulting from iodination could have affected the ability of gD-1(Δ290-299t) to bind to HVEM(200t), thereby influencing the extent of competition (Fig. 8B). Experiments to address the issue of affinity without the use of antibodies and iodinated forms of the glycoproteins are in progress.

Blocking of HSV-1 infection of CHO-HVEM12 cells with forms of gD. After a demonstration that gD binds to HVEM under in vitro conditions, it was of interest to determine whether gD blocked HSV infection of CHO-HVEM12 cells.

Therefore, Vero and CHO-HVEM12 cell monolayers were incubated with increasing concentrations of gD-1(306t) and gD-1(Δ290-299t) at 4°C for 90 min. Then cells were infected with the β-galactosidase reporter virus KOS-gL86 (35, 40). As previously reported (38, 39) and shown in Fig. 9, both forms of gD blocked HSV entry into Vero cells, with gD-1(Δ290-299t) exhibiting enhanced blocking relative to that of gD-1(306t). In the case of CHO-HVEM12 cells, gD-1(Δ290-299t) but not gD-1(306t) blocked infection. The differences seen in blocking by the two forms of gD are consistent with the differences seen in in vitro binding. Another point of interest is that at least 10-fold-more gD-1(Δ290-299t) was needed to inhibit infection of CHO-HVEM12 cells compared with that for Vero cells, consistent with the high probability that Vero and CHO-HVEM12 cells express different levels and kinds of mediators of HSV entry.

Characterization of the gD-HVEM complex by size exclusion chromatography. To study the interaction between gD and HVEM by another method and to assess the stoichiometry of the interaction, we mixed gD-1(Δ290-299t) and HVEM(200t) in solution and examined complex formation by size exclusion chromatography on a Superdex 200 column. Column fractions were analyzed by SDS-PAGE and immunoblotting, probing duplicate blots for gD and HVEM (Fig. 10). The molecular masses of gD-1(Δ290-299t) alone and HVEM(200t) alone were 58 and 40 kDa, respectively (Fig. 10, blots 1). These sizes suggest that both proteins were present in solution in dimeric form. Complex formation was then assessed by mixing various amounts of gD-1(Δ290-299t) with a constant amount of HVEM(200t) to give molar ratios of gD to HVEM of 1:1, 1:2, and 1:3. Each mixture was incubated overnight at 4°C and then applied to a Superdex 200 column. When the proteins were mixed at a 1:1 ratio (Fig. 10, blots 2), approximately 50% of the gD-1(Δ290-299t) and all of the HVEM(200t) eluted in fractions corresponding to a higher molecular mass (113 kDa). These results indicated that the two proteins were in a complex which remained associated during size exclusion chromatography. The presence of a large proportion of free gD-1(Δ290-299t) but not free HVEM(200t) suggested that gD was in excess when the proteins were mixed at a 1:1 ratio, i.e., there was more HVEM than gD in the complex. Alternatively, a

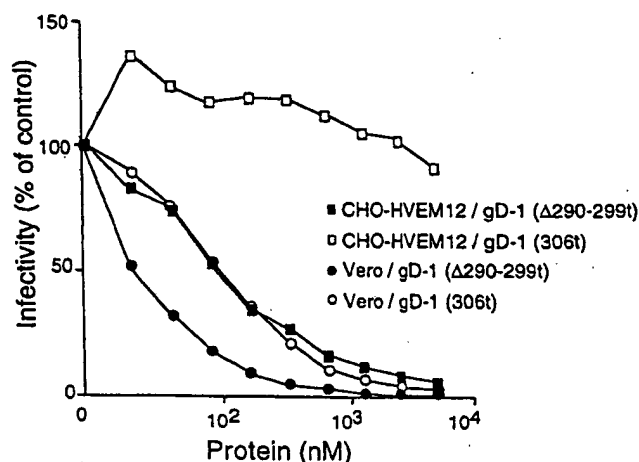


FIG. 9. Effects of soluble gD on infection of Vero and CHO-HVEM12 cells. Cells on 96-well plates were pretreated with various concentrations of gD-1(306t) and gD-1(Δ290-299t) at 4°C for 90 min. KOS-gL86 was then added for an adsorption period of 90 min at 4°C. Then cells were shifted to 37°C for 6 h and lysed for the quantitation of β-galactosidase activity. The data are percentages of β-galactosidase activity detected in the absence of gD.

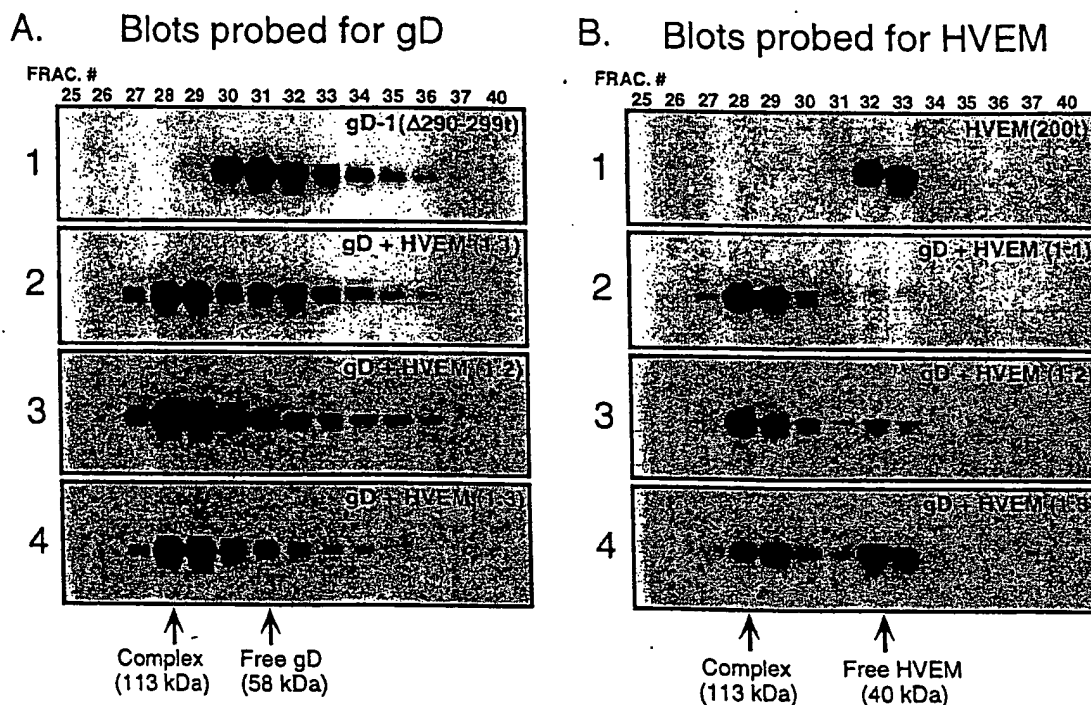


FIG. 10. Gel filtration of the gD-1(Δ290-299t)-HVEM(200t) complex. Samples of gD-1(Δ290-299t) at a concentration of 35 μ M in PBS, HVEM(200t) (35 μ M in PBS), and the two proteins mixed at molar ratios of gD to HVEM of 1:1 (35 μ M each), 1:2 (17.5 μ M gD and 35 μ M HVEM), and 1:3 (11.6 μ M gD and 35 μ M HVEM) were incubated for 16 h at 4°C. Each sample was then chromatographed on a Superdex 200 column. Fractions (FRAC.) of 0.5 ml were collected for each column run and analyzed by SDS-PAGE, followed by Western blotting. (A) Immunoblots were reacted with antiserum R7 to detect gD. Blots: 1, gD-1(Δ290-299t) alone; 2, gD-1(Δ290-299t) and HVEM(200t), mixed at a 1:1 molar ratio; 3, gD-1(Δ290-299t) and HVEM(200t), mixed at a 1:2 molar ratio; 4, gD-1(Δ290-299t) and HVEM(200t), mixed at a 1:3 molar ratio. Arrows indicate the positions of highest A_{280} for the complex and for gD-1(Δ290-299t) run alone. (B) Immunoblots were reacted with antiserum R133 to detect HVEM. Blots: 1, HVEM alone; 2, gD-1(Δ290-299t) and HVEM(200t), mixed at a 1:1 molar ratio; 3, gD-1(Δ290-299t) and HVEM(200t), mixed at a 1:2 molar ratio; 4, gD-1(Δ290-299t) and HVEM(200t), mixed at a 1:3 molar ratio. Arrows indicate the positions of highest A_{280} for the complex and for HVEM(200t) run alone.

fraction of the gD-1(Δ290-299t) was unreactive. However, when the starting concentration of gD was decreased and the amount of HVEM was kept constant prior to mixing, a greater proportion of the total gD was eluted from the column in association with HVEM. At a mixing ratio of 1 mol of gD to 2 mol of HVEM, very little free HVEM or gD-1(Δ290-299t) was seen (Fig. 10, blots 3). At a 1:3 mixing ratio of gD to HVEM, the complex still formed but there was a greater proportion of free HVEM (Fig. 10, blots 4). A simple interpretation of these results is that at a gD/HVEM ratio of 1:2, maximal amounts of both proteins were present as a complex and that this ratio therefore represents the stoichiometry. When the experiment was done with gD-1(306t), no complex was detected by gel filtration (data not shown). This result was not unexpected based on the ELISA data, i.e., the affinity of gD-1(306t) for HVEM(200t) may be too low to maintain a stable complex for the time needed to detect it by gel filtration.

To further evaluate the stoichiometry of the gD-HVEM complex, we carried out SDS-PAGE, followed by silver staining, of fraction 28 (Fig. 10, blots 3) and compared the intensities of the gD and HVEM bands in the complex to the intensities of known concentrations of each protein run separately (Fig. 11). Quantitation of the proteins was carried out by densitometry, and the molar ratio of gD-1(Δ290-299t) to HVEM(200t) was calculated to be 1:2.2.

In addition, we carried out N-terminal sequencing of the complex. As controls, we sequenced unfractionated mixtures of the two proteins at different molar ratios (data not shown). This experiment confirmed the molar ratio of gD-1(Δ290-299t)

to HVEM(200t) (1:2) suggested by gel filtration and SDS-PAGE analysis of the complex (Fig. 10 and 11).

DISCUSSION

Over the past decade, a number of laboratories have endeavored to identify a cellular receptor for HSV by in some cases focusing on potential interactions with gD (3, 4, 22, 28, 34, 36, 47). Most recently, a member of the TNFR family of proteins, HVEM, was identified as a mediator of HSV entry into normally nonpermissive CHO-K1 cells (35). The inability of three strains of HSV with mutations in the gD gene to infect CHO-HVEM12 cells (35) strongly suggested that gD binds directly to HVEM.

If in fact gD does bind to HVEM to mediate virus entry, we should be able to demonstrate that (i) gD interacts specifically with HVEM; (ii) the native conformation, i.e., maintenance of three intact disulfide bonds of gD, may be critical for this interaction (31); (iii) the three N-CHO on gD are not necessary for the HVEM interaction (51, 52); and (iv) gD isolated from the rid and ANG strains of HSV-1 may fail to interact with HVEM (35, 57). The results presented here verify these predictions by showing that purified soluble forms of native gD ectodomain, with or without N-CHO, can bind specifically to a purified form of HVEM ectodomain, whereas denatured gD or rid1 and ANG forms of gD failed to bind. These results demonstrate that gD is a receptor-binding protein for HSV entry into cells via HVEM. Although other glycoproteins, including gB and gH-gL, are also required for entry, their roles

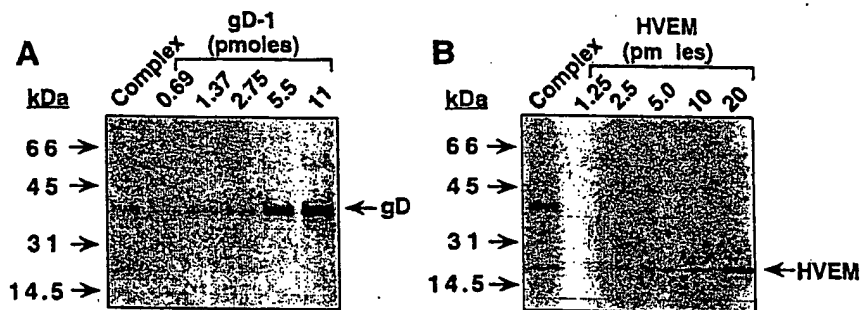


FIG. 11. Analysis of the composition of the complex formed between gD-1(Δ 290-299t) and HVEM(200t). gD-1(Δ 290-299t) at a concentration of 17.5 μ M in PBS was mixed with HVEM(200t) (35 μ M in PBS) and incubated for 16 h at 4°C. The complex was chromatographed on a Superdex 200 column. A portion of the fraction which contained the highest concentration of complex (fraction 28 [Fig. 10, blots 3]) was analyzed by SDS-PAGE, followed by silver staining. (A) Silver-stained gel of the complex (first lane) and of various concentrations of gD-1(Δ 290-299t); (B) silver-stained gel of the complex (first lane) and various concentrations of HVEM(200t). Densitometry was used to construct standard curves of A_{600} versus protein concentration for both proteins, and the amounts of gD and HVEM in the complex were determined from these curves to be 2.7 pmol of gD-1(Δ 290-299t) and 6.0 pmol of HVEM(200t).

do not seem to involve stable interactions with HVEM, at least none that can be detected with isolated soluble forms of these proteins.

Binding of gD to HVEM(200t). To test our predictions, we chose the baculovirus system for producing large amounts of secreted HVEM(200t). Physical-chemical studies confirmed the purity of the nickel-agarose-purified glycoprotein and showed that it is a dimer in solution. Like HVEM:Fc, this protein blocked HSV entry into CHO-HVEM12 cells. We also found that gD-1(306t) bound directly to HVEM(200t) immobilized on an ELISA plate by utilizing antisera to gD to detect the interaction. When the assay was reversed by coating the plate with gD-1(306t) and adding HVEM(200t), we did not detect bound HVEM(200t) with the anti-peptide serum R133. Although the reason for this is not clear, it is possible that residues 139 to 162 of HVEM (corresponding to those used for preparing the antiserum) interact with gD, thereby sterically blocking the binding of the anti-peptide antibodies. Studies to localize the domains of HVEM which interact with gD are in progress.

Our results show that gD-1(306t) bound to HVEM(200t) specifically and in a saturable manner. No other HSV glycoprotein bound to HVEM, and gD did not bind to other soluble receptors. We further found that native gD conformation was critical for its interaction with HVEM but that N-CHO on gD were irrelevant. These two properties meet the criteria for gD function in virus infection. As predicted, forms of gD from the rid1 and ANG strains of HSV-1 did not bind to HVEM(200t). These data suggest that at least residue 27, and perhaps residues 25 and 230 of gD, is directly involved in binding to HVEM. Alternatively, alteration of these residues may indirectly affect the structure of the interacting residues on gD. We previously showed that both gD-1(rid1t) and gD-1(ANGt) exhibited structural differences compared with gD from the KOS strain (38). Interestingly, gD-2(306t) bound to HVEM(200t) as well as gD-1(306t) did, even though they differ in amino acid sequence at 35 residues. Both proteins also block infection of Vero cells by HSV-1 and HSV-2 equally well (39). Significantly, they have the same amino acids at residues 25, 27, and 230 as do the gDs from many strains of HSV-1 and HSV-2.

A gD-1 variant protein carrying a combined deletion and linker-insertion mutation, gD-1(Δ 290-299t), was previously shown to have an enhanced ability to block HSV infection of Vero cells (38, 39). Here we showed that comparable binding to HVEM(200t) occurred at concentrations of gD-1(Δ 290-299t) considerably lower than those of gD-1(306t). Our studies

further indicated that gD-1(Δ 290-299t) and gD-1(306t) competed for the same site(s) on HVEM but that the interaction between gD-1(Δ 290-299t) and HVEM(200t) was stronger. Experiments to examine the affinity of the interaction are in progress. From the results of ELISA experiments, we estimate that the K_d for gD-1(Δ 290-299t) binding to HVEM is in the nanomolar range or less and that the K_d for gD-1(306t) binding to HVEM is in the micromolar range or less. However, these estimates assume that the complex consists of equimolar amounts of gD and HVEM.

In addition to its ability to bind HVEM(200t) in vitro, we found that gD-1(Δ 290-299t) blocked HSV-1 infection of CHO-HVEM12 cells. However, more gD-1(Δ 290-299t) was needed to block infection of CHO-HVEM12 cells than was needed to block Vero cells and gD-1(306t) did not block infection of CHO-HVEM12 cells. One possibility is that CHO-HVEM12 cells overexpress HVEM and that therefore larger amounts of gD-1(306t) (which are not practical to use) are required for inhibition; alternatively, the Vero mediator(s) of HSV entry may bind to both forms of gD with higher affinity than does HVEM. Thus, gD-1(306t) may bind to HVEM on cells with too low an affinity to compete effectively with virion-associated gD. We are in the process of isolating additional HVEM-expressing CHO cell clones to determine whether there is a correlation between the level of HVEM expression and the ability of gD to block infection. It is worth noting that gD-1(Δ 290-299t) was unable to rescue the infectivity of a gD null virus in complementation assays (7). One could argue that enhanced binding of gD to receptor interferes with later steps of viral entry and that a weak interaction, such as that displayed by wild-type gD-1(306t), is beneficial. These possibilities will be addressed in future studies.

Stoichiometry of the gD-HVEM interaction. According to the results of gel filtration experiments, the stoichiometry of the gD-HVEM complex is 1:2. This stoichiometry was further supported by SDS-PAGE analysis as well as by N-terminal sequencing of the fractionated complex. Determination of the mass of the complex by gel filtration (113 kDa) did not permit us to speculate on its exact composition. Assuming that two dimers of HVEM(200t) associate with one dimer of gD-1(Δ 290-299t), the mass of such a complex should be approximately 140 kDa. Alternatively, one dimer of HVEM(200t) complexed with a monomer of gD-1(Δ 290-299t) should have a mass of approximately 75 kDa. Perhaps relevant to our observations, it has been noted that the ectodomain of TNFR migrates anomalously by SDS-PAGE as well as by gel filtration

(30, 43). We are carrying out additional experiments to more accurately determine the size of the complex.

Receptor oligomerization is a mechanism by which many extracellular hormones transmit their signals to the inside of a cell (58, 59). Thus, it is not surprising that a number of receptor-ligand complexes have been shown to contain multiple receptor molecules. Gel filtration studies similar to those used here showed that the ectodomain of the TNFR bound in a 3:3 molar ratio to TNF α (three monomers of TNFR and one trimer of ligand) (43). This ratio is in agreement with that determined from crystallography data (2). A stoichiometry of 1:2 was reported for the complex formed by human growth hormone and the ectodomain of its receptor-binding protein (10). In that case, a sequential binding mechanism that leads to receptor dimerization and cell signalling was postulated. Since the gel filtration studies between gD and HVEM were carried out with mixtures that had been incubated overnight, we would not expect to see 1:1 intermediates even if sequential interaction occurred. Measurements of real-time interaction will be needed to examine this possibility. Whether the 1:2 stoichiometry of the gD-HVEM interaction has significance for subsequent events in HSV infection remains to be examined.

HVEM may be one of several mediators of HSV entry. It was previously noted that the rid and ANG strains of HSV did not exhibit enhanced entry into CHO-HVEM12 cells, although they did infect Vero and HeLa in a normal fashion (35). Moreover, gD-1(rid1t) and gD-1(ANGt) proteins blocked infection of Vero cells by the KOS strain (38) and gD-1(306t) can block infection of Vero cells by many HSV strains, including KOS, but not infection by ANG and rid1 viruses. Here, we found that neither gD-1(rid1t) nor gD-1(ANGt) protein bound to HVEM (200t) in vitro. Experiments are under way to identify molecules which mediate infection by the rid and ANG strains of HSV. Should they work in a fashion analogous to that of HVEM, we would expect them to bind to gD-1(rid1t) and gD-1(ANGt).

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants NS-30606 and NS-36731 from the National Institute of Neurological Diseases and Stroke (R.J.E. and G.H.C.), grant DE-08239 from the National Institute of Dental Research (G.H.C. and R.J.E.), grants AI-18289 (G.H.C. and R.J.E.), AI-36293 (P.G.S.), AI-30040 (J.D.L.), AI-07325 (A.V.N.), and AI-09022 (R.I.M.) from the National Institute of Allergy and Infectious Diseases, and core grants DK-19525 and CA-16520. A portion of this work was also supported by a grant from Wyeth-Lederle Vaccines and Pediatrics (G.H.C. and R.J.E.).

We thank Robert Doms and Paul Bates for providing CD4 and Tva proteins, respectively; Roberto Manservigi for providing gB-providing cells; and Yvonne Shahan and Matthias Ponce de Leon for providing technical assistance.

REFERENCES

- Baker, S. J., and E. P. Reddy. 1996. Transducers of life and death: TNF receptor superfamily and associated proteins. *Oncogene* 12:1-9.
- Banner, D. W., A. D'Arcy, W. Jones, R. Gentz, H.-J. Schoenfeld, C. Broger, H. Loetscher, and W. Lesslauer. 1993. Crystal structure of the soluble human 55 kd receptor-human TNF α complex: implications for the TNF receptor activation. *Cell* 73:431-445.
- Brunetti, C. R., R. L. Burke, B. Hoffack, T. Ludwig, K. S. Dingwell, and D. C. Johnson. 1995. Role of mannose-6-phosphate receptors in herpes simplex virus entry into cells and cell-to-cell transmission. *J. Virol.* 69:3517-3528.
- Brunetti, C. R., R. L. Burke, S. Kornfeld, W. Gregory, F. R. Maslary, K. S. Dingwell, and D. C. Johnson. 1994. Herpes simplex virus glycoprotein D acquires mannose 6-phosphate residues and binds to mannose 6-phosphate receptors. *J. Biol. Chem.* 269:17067-17074.
- Campadelli-Fiume, G., M. Arsenakis, F. Farabegoli, and B. Roizman. 1988. Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. *J. Virol.* 62:159-167.
- Campadelli-Fiume, G., S. Qi, E. Avitabile, L. Foa-Tomasi, R. Brandimarti, and B. Roizman. 1990. Glycoprotein D of herpes simplex virus encodes a domain which precludes penetration of cells expressing the glycoprotein by superinfecting herpes simplex virus. *J. Virol.* 64:6070-6079.
- Chiang, H.-Y., G. H. Cohen, and R. J. Eisenberg. 1994. Identification of functional regions of herpes simplex virus glycoprotein gD by using linker-insertion mutagenesis. *J. Virol.* 68:2529-2543.
- Cohen, G. H., B. Dietzschold, M. Ponce de Leon, D. Long, E. Golub, A. Varrichio, L. Pereira, and R. J. Eisenberg. 1984. Localization and synthesis of an antigenic determinant of herpes simplex virus glycoprotein D that stimulates production of neutralizing antibody. *J. Virol.* 49:102-108.
- Cohen, G. H., M. I. Muggeridge, D. Long, D. A. Sadora, and R. J. Eisenberg. 1992. Structural and functional studies of herpes simplex virus glycoprotein D, p. 217-228. In J. E. Ciarri (ed.), *Genetically engineered vaccines*. Plenum Press, New York, N.Y.
- Cunningham, B. C., M. Ultsch, A. M. de Vos, M. G. Mulkerrin, K. R. Clauser, and J. A. Wells. 1991. Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. *Science* 254:821-825.
- Dean, H. J., S. S. Terhune, M. Shieh, N. Susmarski, and P. G. Spear. 1994. Single amino acid substitutions in gD of herpes simplex virus 1 confer resistance to gD-mediated interference and cause cell-type-dependent alterations in infectivity. *Virology* 199:67-80.
- Dean, H. J., M. S. Warner, S. S. Terhune, R. M. Johnson, and P. G. Spear. 1995. Viral determinants of the variable sensitivity of herpes simplex virus strains to gD-mediated interference. *J. Virol.* 69:5171-5176.
- Dubin, G., N. O. Fishman, R. J. Eisenberg, G. H. Cohen, and H. M. Friedman. 1992. The role of the herpes simplex viruses in immune evasion. p. 111-120. In B. T. Rouse (ed.), *Herpes simplex viruses*. Springer-Verlag, New York, N.Y.
- Dubin, G., and H. Jiang. 1995. Expression of herpes simplex virus type 1 glycoprotein L (gL) in transfected mammalian cells: evidence that gL is not independently anchored to cell membranes. *J. Virol.* 69:4564-4568.
- Eisenberg, R. J., D. Long, D. L. Sadora, H.-Y. Chiang, W. C. Wilcox, W. R. Abrams, M. I. Muggeridge, and G. H. Cohen. 1994. Structure and function of glycoprotein D of herpes simplex virus, p. 43-65. In Y. Becker and G. Darai (ed.), *Frontiers in virology*, vol. 3. Springer-Verlag, Heidelberg, Germany.
- Eisenberg, R. J., M. Ponce de Leon, H. M. Friedman, L. F. Fries, M. M. Frank, J. C. Hastings, and G. H. Cohen. 1987. Complement component C3b binds directly to purified glycoprotein C of herpes simplex virus types 1 and 2. *Microb. Pathog.* 3:423-435.
- Fuller, A. O., and W.-C. Lee. 1992. Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration. *J. Virol.* 66:5002-5012.
- Gilbert, J. M., P. Bates, H. E. Varmus, and J. M. White. 1994. The receptor for the subgroup A avian leukosis sarcoma viruses binds to subgroup A but not to subgroup C envelope glycoprotein. *J. Virol.* 68:5623-5628.
- Herold, B. C., R. J. Visalli, N. Sumarski, C. Brandt, and P. G. Spear. 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulfate and glycoprotein B. *J. Gen. Virol.* 75:1211-1222.
- Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the absorption of virus to cells and in infectivity. *J. Virol.* 65:1090-1098.
- Hillenkamp, F., M. Karas, R. C. Beavis, and B. T. Chait. 1991. Matrix-assisted laser desorption/ionization mass spectrometry of biopolymers. *Anal. Chem.* 63:1192A-1203A.
- Huang, T., and G. Campadelli-Fiume. 1996. Anti-idiotypic antibodies mimicking glycoprotein D of herpes simplex virus identify a cellular protein required for virus spread from cell to cell and virus-induced polykaryocytosis. *Proc. Natl. Acad. Sci. USA* 93:1836-1840.
- Isola, V. J., R. J. Eisenberg, G. R. Siebert, C. J. Hellman, W. C. Wilcox, and G. H. Cohen. 1989. Fine mapping of antigenic site II of herpes simplex virus glycoprotein D. *J. Virol.* 63:2325-2334.
- Izumi, K. M., and J. G. Stevens. 1990. Molecular and biological characterization of a herpes simplex virus type 1 (HSV-1) neuroinvasiveness gene. *J. Exp. Med.* 172:487-496.
- Johnson, D. C., R. L. Burke, and T. Gregory. 1990. Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. *J. Virol.* 64:2569-2576.
- Johnson, D. C., and M. W. Ligas. 1988. Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. *J. Virol.* 62:4605-4612.
- Johnson, R. M., and P. G. Spear. 1989. Herpes simplex virus glycoprotein D mediates interference with herpes simplex virus infection. *J. Virol.* 63:819-827.
- Kaner, R. J., A. Baird, A. Mansukhani, C. Basilico, B. D. Summers, R. Z. Florkiewicz, and D. P. Hajjar. 1990. Fibroblast growth factor receptor is a portal of cellular entry for herpes simplex virus type 1. *Science* 248:1410-1413.
- Lee, W.-C., and A. O. Fuller. 1993. Herpes simplex virus type 1 and pseudorabies virus bind to a common saturable receptor on Vero cells that is not

- heparan sulfate. *J. Virol.* 67:5088-5097.
30. Loetscher, H., R. Gentz, M. Zulauf, A. Lustig, H. Tabuchi, E.-J. Schlager, M. Brockhurst, H. Gallati, M. Manneberg, and W. Lesslauer. 1991. Recombinant 55-kDa tumor necrosis factor (TNF) receptor. *J. Biol. Chem.* 266:18324-18329.
 31. Long, D., W. C. Wilcox, W. R. Abrams, G. H. Cohen, and R. J. Eisenberg. 1992. Disulfide bond structure of glycoprotein D of herpes simplex virus types 1 and 2. *J. Virol.* 66:6668-6685.
 32. Manservigi, R., M. P. Grossi, R. Gualandri, P. G. Balboni, A. Marchini, A. Rotola, P. Rimessi, D. Di Luca, E. Cassal, and G. Barbanti-Brodano. 1990. Protection from herpes simplex virus type 1 lethal and latent infections by secreted recombinant glycoprotein B constitutively expressed in human cells with a BK virus episomal vector. *J. Virol.* 64:431-436.
 33. Manservigi, R., R. Gualandri, M. Negrini, L. Albonici, G. Milanese, E. Cassal, and G. Barbanti-Brodano. 1988. Constitutive expression in human cells of herpes simplex virus type 1 glycoprotein B gene cloned in an episomal eukaryotic vector. *Virology* 167:284-288.
 34. Mirda, D. P., D. Navarro, P. L. Lee, P. Paz, L. Pereira, and L. T. Williams. 1992. The fibroblast growth factor receptor is not required for herpes simplex virus type 1 infection. *J. Virol.* 66:448-457.
 35. Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* 87:427-436.
 36. Muggeridge, M. I., G. H. Cohen, and R. J. Eisenberg. 1992. Herpes simplex virus infection can occur without involvement of the fibroblast growth factor receptor. *J. Virol.* 66:824-830.
 37. Muggeridge, M. I., W. C. Wilcox, G. H. Cohen, and R. J. Eisenberg. 1990. Identification of a site on herpes simplex virus type 1 gD that is essential for infectivity. *J. Virol.* 64:3617-3626.
 38. Nicola, A. V., C. Peng, H. Lou, G. H. Cohen, and R. J. Eisenberg. 1997. Antigenic structure of soluble herpes simplex virus (HSV) glycoprotein D correlates with inhibition of HSV infection. *J. Virol.* 71:2940-2946.
 39. Nicola, A. V., S. H. Willis, N. N. Naidoo, R. J. Eisenberg, and G. H. Cohen. 1996. Structure-function analysis of soluble forms of herpes simplex virus glycoprotein D. *J. Virol.* 70:3815-3822.
 40. Novotny, M. J., and P. G. Spear. Unpublished data.
 41. Peng, T., G. Dubin, R. J. Eisenberg, and G. H. Cohen. Unpublished data.
 42. Peng, T., M. Ponce de Leon, H. Jiang, G. Dubin, G. H. Cohen, and R. J. Eisenberg. 1996. Studies of a soluble form of the HSV gH-gL complex expressed by a mammalian cell line; abstr. 130. *In* 21st International Herpesvirus Workshop, De Kalb, Ill.
 43. Pennica, D., V. T. Lam, R. F. Weber, W. J. Kohr, L. J. Basa, M. W. Spellman, A. Ashkenazi, S. J. Shire, and D. V. Goeddel. 1993. Biochemical characterization of the extracellular domain of the 75-kilodalton tumor necrosis factor receptor. *Biochemistry* 32:3131-3138.
 44. Roller, R. J., and B. Roizman. 1994. A herpes simplex virus US11-expressing cell line is resistant to herpes simplex virus infection at a step in viral entry mediated by glycoprotein D. *J. Virol.* 68:2830-2839.
 45. Rux, A. H., W. T. Moore, J. D. Lambris, W. R. Abrams, C. Peng, H. M. Friedman, G. H. Cohen, and R. J. Eisenberg. 1996. Disulfide bond structure determination and biochemical analysis of glycoprotein C from herpes simplex virus. *J. Virol.* 70:5455-5465.
 46. Rux, J. J., S. H. Willis, A. V. Nicola, R. M. Burnett, G. H. Cohen, and R. J. Eisenberg. 1996. Crystallization of truncated HSV glycoprotein D produced in a baculovirus expression system; abstr. 119. *In* 21st International Herpesvirus Workshop, De Kalb, Ill.
 47. Shieh, M.-T., and P. G. Spear. 1991. Fibroblast growth factor receptor: does it have a role in the binding of herpes simplex virus? *Science* 253:208-209.
 48. Showalter, S. D., M. Zweig, and B. Hampar. 1981. Monoclonal antibodies to herpes simplex virus type 1 proteins, including the immediate-early protein ICP 4. *Infect. Immun.* 34:684-692.
 49. Sisk, W. P., J. D. Bradley, R. J. Leipold, A. M. Stoltzfus, M. Ponce de Leon, M. Hilf, C. Peng, G. H. Cohen, and R. J. Eisenberg. 1994. High-level expression and purification of secreted forms of herpes simplex virus type 1 glycoprotein gD synthesized by baculovirus-infected insect cells. *J. Virol.* 68:766-775.
 50. Smith, C. A., T. Farrar, and R. G. Goodwin. 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell* 76:959-962.
 51. Sodor, D. L., G. H. Cohen, M. I. Muggeridge, and R. J. Eisenberg. 1991. Absence of asparagine-linked oligosaccharides from glycoprotein D of herpes simplex virus type 1 results in a structurally altered but biologically active protein. *J. Virol.* 65:4424-4431.
 52. Sodor, D. L., R. J. Eisenberg, and G. H. Cohen. 1991. Characterization of a recombinant herpes simplex virus which expresses a glycoprotein D lacking asparagine-linked oligosaccharides. *J. Virol.* 65:4432-4441.
 53. Spear, P. G. 1993. Membrane fusion induced by herpes simplex virus, p. 201-232. *In* J. Bentz (ed.), *Viral fusion mechanisms*. CRC Press, Inc., Boca Raton, Fla.
 54. Tal-Singer, R., R. J. Eisenberg, T. Valyi-Nagy, N. W. Fraser, and G. H. Cohen. 1994. N-linked oligosaccharides on herpes simplex virus glycoprotein gD are not essential for establishment of viral latency or reactivation in the mouse eye model. *Virology* 202:1050-1053.
 55. Tal-Singer, R., C. Peng, M. Ponce de Leon, W. R. Abrams, B. W. Banfield, F. Tufano, G. H. Cohen, and R. J. Eisenberg. 1995. The interaction of herpes simplex virus glycoprotein gC with mammalian cell surface molecules. *J. Virol.* 69:4471-4483.
 56. Tessier, D. C., D. Y. Thomas, H. E. Khouri, F. Laliberte, and T. Vernet. 1991. Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide. *Gene* 98:177-183.
 57. Warner, M. S., R. I. Montgomery, and P. G. Spear. 1996. A herpes simplex virus type 1 mutant resistant to gD mediated interference is altered in utilization of cell surface receptors for entry, p. 94, abstr. W7-3. *In* American Society for Virology Annual Meeting, London, Ontario, Canada.
 58. Wells, J. A. 1996. Binding in the growth hormone complex. *Proc. Natl. Acad. Sci. USA* 93:1-6.
 59. Wells, J. A. 1994. Structural and functional basis for hormone binding and receptor oligomerization. *Curr. Opin. Cell Biol.* 6:163-173.
 60. Willis, S. H., A. V. Nicola, J. J. Rux, R. M. Burnett, R. J. Eisenberg, and G. H. Cohen. Unpublished data.
 61. Willis, S. H., C. Peng, M. Ponce de Leon, A. V. Nicola, A. H. Rux, G. H. Cohen, and R. J. Eisenberg. Expression and purification of secreted forms of herpes simplex virus glycoproteins from baculovirus-infected insect cells. *In* M. S. Brown and A. R. MacLean (ed.), *Methods in molecular medicine*, in press. Humana Press, Totowa, N.J.
 62. WuDunn, D., and P. G. Spear. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* 63:52-58.

The Major Neutralizing Antigenic Site on Herpes Simplex Virus Glycoprotein D Overlaps a Receptor-Binding Domain

J. CHARLES WHITBECK,^{1,2,3*} MARTIN I. MUGGERIDGE,^{1,2†} ANN H. RUX,^{1,2,3} WANGFANG HOU,^{1,2}
CLAUDE KRUMMENACHER,^{1,2} HUAN LOU,^{1,2} ALBERT VAN GEELLEN,^{1‡}
ROSELYN J. EISENBERG,^{2,3} AND GARY H. COHEN^{1,2}

*School of Dental Medicine,¹ Center for Oral Health Research,² and School of Veterinary Medicine,³
University of Pennsylvania, Philadelphia, Pennsylvania 19104*

Received 28 May 1999/Accepted 24 August 1999

Herpes simplex virus (HSV) entry is dependent on the interaction of virion glycoprotein D (gD) with one of several cellular receptors. We previously showed that gD binds specifically to two structurally dissimilar receptors, HveA and HveC. We have continued our studies by using (i) a panel of baculovirus-produced gD molecules with various C-terminal truncations and (ii) a series of gD mutants with nonoverlapping 3-amino-acid deletions between residues 222 and 254. Binding of the potent neutralizing monoclonal antibody (MAb) DL11 (group Ib) was unaffected in forms of gD containing residues 1 to 250 but was greatly diminished in molecules truncated at residue 240 or 234. Both receptor binding and blocking of HSV infection were also affected by these C-terminal truncations. gD-1(234t) bound weakly to both HveA and HveC as determined by enzyme-linked immunosorbent assay (ELISA) and failed to block infection. Interestingly, gD-1(240t) bound well to both receptors but blocked infection poorly, indicating that receptor binding as measured by ELISA is not the only gD function required for blocking. Optical biosensor studies showed that while gD-1(240t) bound HveC with an affinity similar to that of gD-1(306t), the rates of complex formation and dissociation were significantly faster than for gD-1(306t). Complementation analysis showed that any 3-amino-acid deletion between residues 222 and 251 of gD resulted in a nonfunctional protein. Among this set of proteins, three had lost DL11 reactivity (those with deletions between residues 222 and 230). One of these proteins (deletion 222-224) was expressed as a soluble form in the baculovirus system. This protein did not react with DL11, bound to both HveA and HveC poorly as shown by ELISA, and failed to block HSV infection. Since this protein was bound by several other MAbs that recognize discontinuous epitopes, we conclude that residues 222 to 224 are critical for gD function. We propose that the potent virus-neutralizing activity of DL11 (and other group Ib MAbs) likely reflects an overlap between its epitope and a receptor-binding domain of gD.

The herpes simplex virus (HSV) genome codes for at least 11 glycoproteins, most of which are detectable in the virion envelope (50). Infection of susceptible cells is initiated by the attachment of virions, via glycoprotein C (gC) and/or gB, to cell surface heparan sulfate proteoglycans (21, 22, 59). This is followed by the interaction of gD with a cellular receptor. Then, pH independent fusion occurs between the virus envelope and the host cell plasma membrane (58); gB, gD, and the gH-gL complex have all been implicated in this step (50, 52).

Recently, expression cloning was used to identify several human genes whose products convert the normally nonpermissive Chinese hamster ovary cells into cells that are permissive for HSV type 1 (HSV-1) and HSV-2 entry (9, 19, 40, 53). These mediators of HSV entry are known as HveA, HveB, and HveC. HveA is a member of the tumor necrosis factor receptor superfamily of proteins (40) and interacts with both lymphotoxin α and LIGHT (38). HveB (also called PRR2) and HveC (also called PRR1) are closely related members of the immunoglobulin superfamily of proteins (36.1% amino acid sequence identity within the predicted extracellular domains) which share

53.2 and 33.9% amino acid sequence identities, respectively, with the poliovirus receptor extracellular domain (14, 19, 37, 53). The normal cellular functions of these proteins remain unknown, although recent data suggest that the murine homolog of HveB may be a cell-cell adhesion molecule (1). A splice variant of HveC, called HlgR, can also mediate HSV infection of nonpermissive cells (9). Soluble forms of gD have been shown to bind directly to soluble forms of HveA, HveC, and HlgR but not to HveB (8, 9, 31, 54, 55). In addition, antibodies to the receptors have been shown to block infection by HSV (9, 40, 53). Thus, it is clear that HSV can utilize several different and structurally unrelated cell surface proteins as receptors and that two of these receptors bind directly to HSV gD.

Two approaches were used in previous studies to try to define the relationship between gD structure and function: (i) examination of the properties of a panel of monoclonal antibodies (MAbs) to gD (11, 12, 23, 41, 43) and (ii) examination of the properties of a panel of gD mutants (7, 17, 42). First, the antigenic site I of gD was defined by seven MAbs, all of which possess potent virus-neutralizing activity in the absence of complement (41). Although all group I MAbs block the binding of other group I antibodies to gD, further subdivision of these MAbs into groups Ia and Ib was done on the basis of studies with truncated and other mutant forms of gD. Two group Ia MAbs, HD1 and LP2 (11), bind to gD truncated at amino acid residue 233, whereas DL11 and four other group Ib antibodies do not (11, 43). More recently, we showed that, whereas DL11 blocks the binding of soluble HveA or HveC to

* Corresponding author. Mailing address: 212 Levy Bldg., School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104. Phone: (215) 898-6553. Fax: (215) 898-8385. E-mail: whitbeck@biochem.dental.upenn.edu.

† Present address: Department of Microbiology and Immunology, Louisiana State University School of Medicine, Shreveport, LA 71130.

‡ Present address: Department of Microbiology, University of Nevada at Reno, Reno, NV 89557.

HSV virions, HD1 blocks the binding of HveC but not of HveA to HSV (31, 44). On the other hand, MAbs in group VII blocked the binding of HveA but not of HveC to HSV (31, 44). Taken together, these results suggest that the binding of gD to each of these receptors involves both a common region as well as unique portions of the gD molecule. Furthermore, information about the location of epitopes within antigenic sites I and VII has provided important clues as to the portions of gD involved in the binding of each receptor. For example, since group VII MAbs recognize a continuous epitope within amino acids 11 to 19 (10, 26), it is likely that residues near the amino terminus of gD are important for its interaction with HveA. In support of this hypothesis, a mutant form of gD with a change at amino acid 27 fails to bind to HveA but still interacts with HveC (31, 54). Not surprisingly, viruses with this change in gD are unable to utilize HveA as an entry receptor (40).

Using gD mutants and complementation analysis, we previously identified four distinct regions within the gD primary structure that are important for HSV infection which were designated functional regions I through IV (7). Several observations can be made regarding the relationship between antigenic site Ib (discussed above) and functional regions II and III. First, all of the linker insertions within functional region II abolished or greatly diminished binding by the group Ib MAbs, DL11. Second, functional region II (residues 125 to 161) encompasses residues previously shown to affect the binding of certain group Ib antibodies (residues 132 and 140). Third, functional region III (residues 225 to 246) includes residues known to be required for group Ib antibody binding. These observations taken together suggest that functional regions II and III may be closely positioned within the folded (tertiary) structure of gD and may, together, form a functional domain. Here we address the contribution of these two regions to the

Here we address the contribution of gD residues between 222 and 275 to the formation of both antigenic site Ib and a functional (receptor-binding) domain. To accomplish this, we constructed two sets of HSV-1 gD mutants. The first group is a nested set of C-terminal truncations consisting of molecules truncated at residues 234, 240, 250, 260, 285, and 306. The second set of constructs is a panel of 11 gD mutants containing adjacent, nonoverlapping, 3-amino-acid deletions within functional region III. Our results support our hypothesis that there is an overlap between antigenic site Ib and a domain involved in binding to the HSV receptors, HveA and HveC.

MATERIALS AND METHODS

Cells and virus. HeLa and Vero cells were obtained from the ATCC and grown in Dulbecco modified Eagle medium (DMEM; Gibco) supplemented with 5% fetal bovine serum (FBS). Sf9 (*Spodoptera frugiperda*) cells (GIBCO BRL) were grown in SF900II medium (GIBCO BRL). The HSV1(KOS) recombinant, hrR3 (20), in which the *lacZ* gene, under the control of the ICP6 promoter, has been inserted into the ICP6 locus, was propagated in D14 cells as described by Goldstein and Weller (20) and titers were determined on Vero cells. COS-1 cells were grown in DMEM supplemented with 5% FBS. VD60 cells were grown in DMEM containing 5% FBS and 1 mM histidinol (35). The isolation and propagation of the gD-null virus, F-gDB, has been described previously (35). The HSV-1 strain KOS was used where indicated.

Construction of baculovirus recombinants expressing truncated forms of gD. The strategy employed in the construction of a baculovirus recombinant expressing gD-1(306r) has been described in detail elsewhere (49). The construction of bac-gD-1(285t) and bac-gD-1(234t) has also been described (47). Briefly, PCR primers were synthesized in order to amplify and modify the gD ectodomain coding region for cloning into the pVT-Bac transfer vector plasmid and expression in a recombinant baculovirus. The upstream primer, 5'-TTTTCGATCCC AATATGCGCTTGGCGGATG-3', hybridized to the noncoding strand of the gD open reading frame (ORF) immediately beyond the predicted signal sequence coding region and incorporated a *Bam*HI restriction enzyme cleavage site (underlined). Three different downstream primers were used separately with the upstream primer to generate ORFs coding for gD truncated after residue 260, 250, or 240. The downstream primer used to amplify the PCR fragment for gD-1(260t) cloning and expression was 5'-GGCGAATTCAGTGGTGGTGGT

GGTGGTGGGTCTCGGACAGCTCCGGGGGCAG-3' and incorporated an *EcoRI* restriction enzyme cleavage site (underlined). The downstream primer used to amplify the PCR fragment for gD-1(250t) cloning and expression was 5'-GCGCAATTCACTGGTGTTGGTGGTGGCTCGTATGGGGCC TT-3' and incorporated an *EcoRI* restriction enzyme cleavage site (underlined). The downstream primer used to generate the PCR fragment for gD-1(240t) cloning and expression was 5'-GCGCAATTCACTGGTGTTGGTGGTGGTGGTGGTCCC GGCGCATCTCAAGCTGTATA-3' and incorporated an *EcoRI* restriction enzyme cleavage site (underlined). The primer used to generate the PCR fragment for gD-1(A222-224, 306t) cloning and expression was 5'-TTTTCTGC AGTTAATGATGATGATGATGATGGAAGGCGTCGCGG-3' and incorporated a *PstI* restriction enzyme cleavage site (underlined). The PCR-amplified DNA fragments coded for gD lacking its natural signal sequence so that the melittin signal sequence, coded for by pVT-Bac, would replace the missing gD signal sequence. The downstream PCR primers were also designed to append six histidine codons prior to the termination codon to allow for purification of the recombinant proteins by nickel agarose chromatography. The PCR-amplified products were then digested with *BamHI* and either *EcoRI* or *PstI* and cloned into pVT-Bac which had been digested with the same enzymes. Once cloned into pVT-Bac, the resulting plasmid constructs were cotransfected with baculovirus DNA (Baculogold; Pharmingen) into Sf9 cells growing in monolayer culture. After 4 days, the culture supernatant (containing recombinant progeny virus) was replated onto Sf9 cell monolayers under Grace's insect cell medium containing 1% agarose. Recombinant virus plaques were picked, amplified, and screened for the expression of secreted gD by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis of the culture medium from Sf9 cells infected with recombinant virus picks. Virus clones expressing gD were plaque purified two times, and protein expression from individual virus clones was verified at each stage by SDS-PAGE and immunoblot analysis. The plaque-purified baculovirus recombinant selected for routine use in production of gD-1 truncated at residue 260 was named bac-gD-1(260t). The soluble protein produced by bac-gD-1(260t) is referred to as gd-1(260t). The nomenclature for the 250 and 240 truncations followed the same pattern. These designations indicate that the secreted gD produced is truncated after the indicated amino acid residue of the predicted mature (signal sequence removed) protein (In this numbering system, the initiator methionine residue of gD occurs at position -25).

Production and purification of recombinant baculovirus-produced proteins. Production and purification of gD-1(306t), gD-1(285t), gD-1(234t), HveA(200t), and HveC(346t) have been described (31, 47, 49, 54, 56). Production and purification of gD-1(260t), gD-1(250t), gD-1(240t), and gD-1(Δ222-224, 306t) were carried out as described previously for HveA(200t) [also called HVEM(200t) (54)].

ELISA. Soluble receptor proteins HveA(200t) or HveC(346t) in phosphate-buffered saline (PBS) were bound to 96-well enzyme-linked immunosorbent assay (ELISA) plates for 3 h at room temperature. The plates were washed three times with PBS-0.2% Tween 20 and incubated in blocking solution (PBS, 5% nonfat milk, 0.2% Tween 20) for 30 min at 25°C. Plates were then washed three times with PBS-0.2% Tween 20 and incubated with truncated forms of gD at various concentrations in blocking solution for 16 h at 4°C. Plates were then washed three times with PBS-0.2% Tween 20 and incubated for 30 min with R7 (a rabbit polyclonal antiserum against gD) diluted 1 to 1,000 in blocking solution. After three washes with PBS-0.2% Tween 20, the plates were incubated in horseradish peroxidase-conjugated goat anti-rabbit antibody (Boehringer Mannheim) diluted 1/1,000 in blocking solution. Plates were washed three times with PBS-0.2% Tween 20 and then once with 20 mM sodium citrate (pH 4.5). After removal of the citrate buffer, ABTS substrate solution (Moss, Inc.) was added, and the absorbance at 405 nm in individual wells was read by using a Perkin-Elmer HTS 7000 Bio-Assay Reader. Finally, absorbance was plotted against the concentration of gD used.

Antibodies. R7 is a rabbit polyclonal antiserum raised against native, full-length gD-2 isolated from virus-infected cells (26). R69 is a rabbit polyclonal antiserum raised against denatured, full-length gB-1 isolated from virus-infected cells (16). 1D3 is a group VII MAb recognizing gD residues 11 to 19 (13, 18). DL6 is a group II MAb recognizing residues 272 to 279 (15, 26). MAbs HD1 (group Ia), DL11 (group Ib), D2 (group Ib), DL2 (group VI), and ABD (group III) recognize discontinuous epitopes (11, 23, 41, 46, 48).

blocking of HSV-1 entry into mammalian cells with soluble proteins. The blocking of HSV entry into cells with soluble gD was carried out as described previously (27) and as modified by Nicola et al. (45).

SDS-PAGE. Purified glycoproteins were separated by SDS-PAGE under "native" (0.1% SDS, no reducing agent, no boiling [11]) or denaturing (samples boiled 10 min in 2.5% SDS–350 mM β -mercaptoethanol) conditions in precast Tris-glycine gels (Novex). After SDS-PAGE, separated proteins were stained with silver nitrate (Pharmacia) or transferred to nitrocellulose, probed with antibodies, and visualized by enhanced chemiluminescence (Amersham).

Construction of gD-1 3-amino-acid deletion series. Oligonucleotide-directed mutagenesis was carried out by using the method of Zoller and Smith (60), as modified by Kunkel et al. (33, 34), to generate the series of plasmid constructs expressing gD containing sequential, nonoverlapping 3-amino-acid deletions spanning residues 222 through 254. The template for mutagenesis was an M13mp18 construct containing the gD-1 (Patton) ORF (cloned into the unique

*Hind*III site) which was excised from plasmid pRE4 (12) by *Hind*III digestion. The specific mutagenic primers used were as follows: $\Delta 222-224$, 5'-TGGTCTCGGGGGCAGCATC-3'; $\Delta 225-227$, 5'-ACGGTGCCTGGATGAAGCGG GGC-3'; $\Delta 228-230$, 5'-GTATACGGCGACGTTCTCGGGGAT-3'; $\Delta 231-233$, 5'-CTTCAAGCTGTAGGTGCGTGGTT-3'; $\Delta 234-236$, 5'-CCCGGCGATCTTACGGCGACGGT-3'; $\Delta 237-239$, 5'-CCCGTGCCACCCCAAGCTGTATA C-3'; $\Delta 240-242$, 5'-GGCCTTGGGCGCGGCGATCTTCAAGC-3'; $\Delta 243-245$, 5'-CGTGTATGGGCGTGCCACCCGCG-3'; $\Delta 246-248$, 5'-CAGGGTGCTC GTCITGGGCCCCGTGC-3'; $\Delta 249-251$, 5'-TCCGGGGGCGAGCAGGTATGG GCCTT-3'; $\Delta 252-254$, 5'-GGACAGCTCCGGGGTGCTCGTGTAT-3'.

After mutagenesis, the gD ORFs were excised (*Hind*III) from M13 replicative-form DNA and transferred into the mammalian expression plasmid, pRSVnt-EPA (5). Plasmids containing the gD ORF in the desired orientation were sequenced by using the method of Chen and Seeburg (6) to confirm the presence of the anticipated mutations (nine nucleotide deletions) within the gD ORF.

Antigenic analysis of mutants. Transfection of COS cells and the subsequent preparation of cytoplasmic extracts were performed as previously described (12, 41).

Immunoperoxidase staining. This procedure, which was performed as previously described (43), is a modification of that of Holland et al. (24) and Kousoulas et al. (30). Surface staining of transfected cells was studied with unfixed cells; for detection of intracellular antigens, the cells were fixed with 5% methanol in PBS before incubation with the MAbs.

Complementation assay. The assay was performed essentially as previously described (43), except that COS cells were used instead of Vero cells. Briefly, cells were transfected with DNA-calcium phosphate precipitates for 16 h at 37°C and then washed and incubated in DMEM-5% FBS for 8 h at 37°C. Each dish of cells was subsequently infected at room temperature with 10^6 PFU of F-gD β virus, followed by the addition of 5 ml of DMEM-5% FBS and incubation for 1 h at 37°C. The medium was then removed, and extracellular virus was inactivated by incubating the monolayer for 1 min in glycine-saline (pH 3.0) (4, 25). After 18 h in DMEM-5% FBS at 37°C, the medium was removed and stored at -70°C for subsequent determination of the virus titers. The cells were lysed by freeze-thawing and sonication with a Microson cell disruptor. Nuclei were then pelleted by low-speed centrifugation, and the supernatant was stored at -70°C for subsequent determination of the virus titers. Both intracellular and extracellular virus titers were determined on VD60 cells. Transfection with salmon sperm DNA was used as the negative control. One hundred percent complementation is defined as the titer obtained after transfection with plasmid pRE4, which expresses wild-type gD (12). Complementation with a mutant is then defined by the following formula: % complementation = $100 \times (\text{titer with mutant plasmid} - \text{titer with carrier DNA}) / (\text{titer with pRE4} - \text{titer with carrier DNA})$.

Optical biosensor experiments. Biosensor experiments were carried out on a Biacore X optical biosensor (Biacore AB) at 25°C as previously described (32, 47). Biosensor data were analyzed by using a global fitting routine with BIA-evaluation software, version 3.0 (2). Model curve fitting was carried out by using a 1:1 Langmuir interaction with drifting baseline. This models the simple interaction between ligand (L) and receptor (R) as follows: $L + R \rightleftharpoons LR$. The rate of association (k_{on}) was measured from the forward reaction, and k_{off} was measured from the reverse reaction. For gD-1(234t), a maximum k_{on} was estimated as previously described (47) by using the equation $\ln(R_0/R_n) = k_{on}(t_n - t_0)$, where R_0 is the response at time zero (t_0) of dissociation and R_n is the response at time n (t_n) (29). Scatchard analyses of the gD-1(234t)-receptor complexes were performed as previously described (47).

RESULTS

C-terminal truncations. Krummenacher et al. (31) and Rux et al. (47) showed that, compared to gD truncated at residue 306 [gD-1(306t)], molecules truncated at residues 285 [gD-1(285t)] and 275 [gD-1(275t)] exhibited enhanced receptor binding. In contrast, a form consisting of residues 1 to 234 [gD-1(234t)] exhibited greatly diminished receptor binding. gD-1(234t) was shown to retain much of the native structure of the full-length molecule in that most MAbs recognizing discontinuous epitopes of gD reacted with gD-1(234t) (47). One exception was that the group Ib MAb, DL11, bound poorly to gD-1(234t). Since gD-1(234t) lacks a significant portion of functional region III (7) (residues 225 to 246), we reasoned that the diminished receptor binding of gD-1(234t) was consistent with the idea that functional region III is directly involved in receptor binding. To define more precisely the C-terminal gD residues required for receptor binding as well as for the binding of MAb DL11, we expressed three additional forms of gD in the baculovirus system. These gD molecules were truncated after residues 260 [gD-1(260t)], 250 [gD-1(250t)], and 240 [gD-1(240t)]. Stick diagrams of these, as well

as other recombinant baculovirus products, are shown in Fig. 1A. Each truncated form of gD was constructed such that six histidine residues were present at the C terminus to allow for purification by nickel chromatography. The gD truncation mutants were purified by immunoaffinity chromatography [gD-1(306t) and gD-1(285t)] or by nickel chromatography (all other forms of gD). To assess the purity of the recombinant proteins, similar amounts were loaded onto an SDS-10% polyacrylamide gel, electrophoresed, and stained with silver nitrate. All of the proteins were purified to near homogeneity and were of the expected sizes (Fig. 1B). Western blot analysis with the group VII MAb 1D3 (Fig. 1C) confirmed that all of these purified proteins retained the correct N terminus of gD (Fig. 1A).

Antigenic analysis of C-terminal gD truncations. To assess the antigenic structure of the recombinant gD molecules, the proteins were separated on nondenaturing ("native") SDS-polyacrylamide gels, and blots were probed with various MAbs. The blot shown in Fig. 2A was reacted with the group II MAb DL6, which recognizes a linear epitope (residues 272 to 279) (26) (Fig. 1A). The expected pattern of reactivity with DL6 was observed in that proteins smaller than gD-1(285t) were not reactive. The blots shown in Fig. 2B to D were reacted with MAbs DL2, ABD, and HD1, each of which recognizes a separate discontinuous epitope on gD. All of the truncated proteins reacted similarly with these MAbs, indicating that the native structure of gD was not grossly altered by the truncations. The blots shown in Fig. 2E and F were reacted with two group Ib MAbs, DL11 and D2. Although DL11 bound strongly to gD-1(306t), gD-1(285t), gD-1(260t), and gD-1(250t), it bound weakly to gD-1(240t) and gD-1(234t). In previous studies, we showed that DL11 competed with soluble HveA and HveC for binding to gD in HSV virions, suggesting that it binds within or near a region of gD involved in receptor interaction (31, 44). According to the data presented in Fig. 2E, gD residues immediately upstream of 250 contribute to the DL11 epitope. MAb D2, like DL11, bound weakly to gD-1(240t) and gD-1(234t) but also exhibited reduced reactivity with gD-1(250t) when compared with molecules truncated after residues 260, 285, and 306. These results confirm and extend previous work mapping residues critical to the formation of antigenic site Ib (11, 12, 41-43).

ELISA. Previous studies showed that, compared to gD-1(306t), molecules truncated after residues 285 and 275 bound to HveA and HveC with increased affinity, while a molecule truncated after residue 234 bound with reduced affinity (31, 47). To assess the effect of the C-terminal truncations on receptor interaction, we analyzed their binding to truncated forms of HveA [HVEM(200t)] and [HveC (HveC(346t))] by ELISA (Fig. 3). Figure 3A shows the binding of truncated forms of gD to HveA, while Fig. 3B shows their binding to HveC. As previously reported (31, 47), gD-1(285t) bound to both HveA and HveC, as seen by ELISA, ca. 100-fold better than did gD-1(306t). gD-1(260t) and gD-1(250t) bound to both receptors as well as gD-1(285t). However, gD-1(240t) bound to both receptors about as well as gD-1(306t), whereas the binding of gD-1(234t) was nearly undetectable. We conclude from these observations that gD residues between positions 234 and 240 are critical for receptor binding and that residues between positions 240 and 250 may also be involved (since the receptor-binding activity of gD-1(240t), though not eliminated, is reduced relative to larger forms of gD). It is of interest to note that gD-1(240t), which showed diminished reactivity with the MAb DL11, still bound to both receptors.

Biosensor analysis of gD binding to HveA and HveC. Previously, we used optical biosensor technology to show that the increased affinity of gD-1(285t) for both HveA and HveC rel-

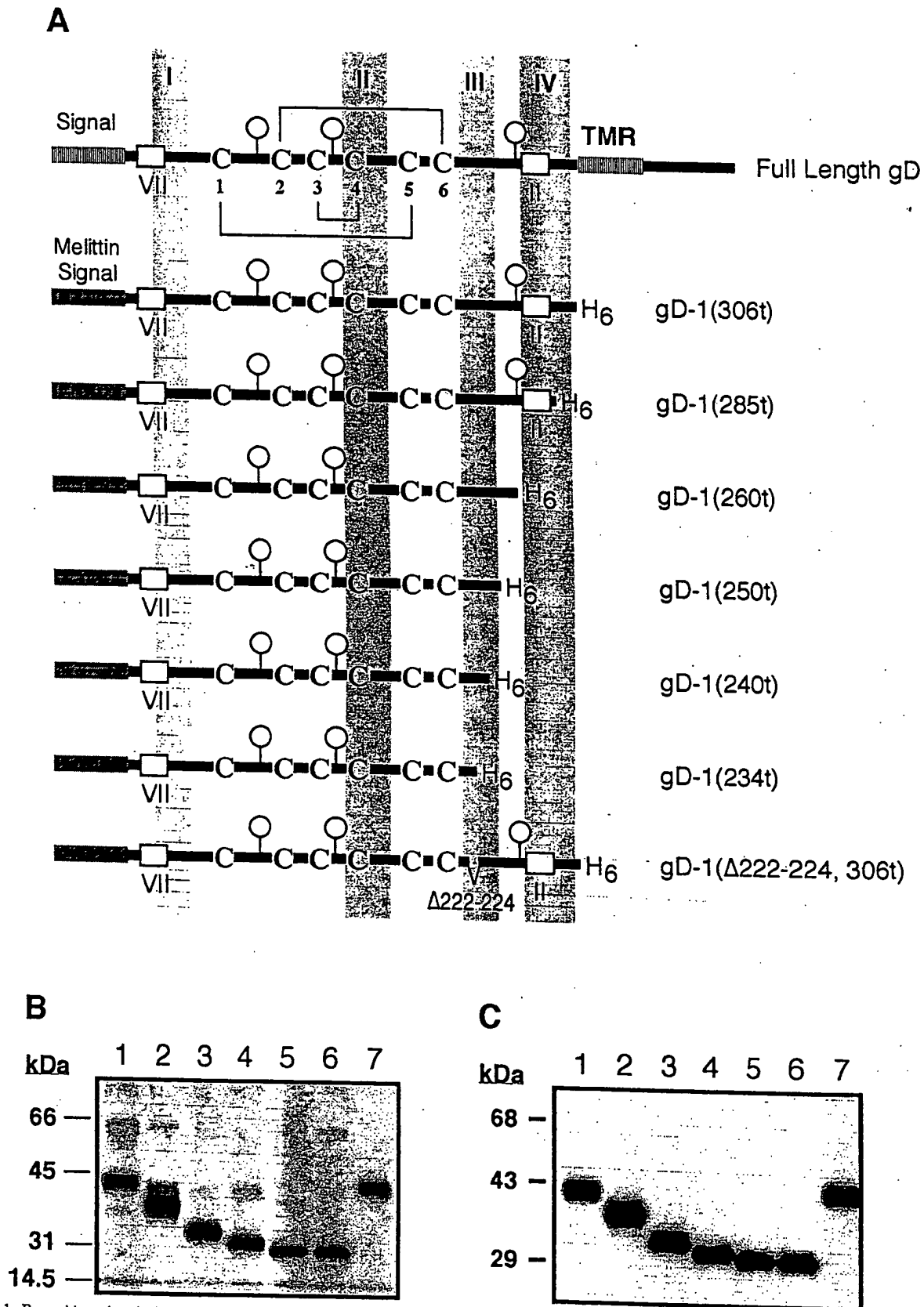


FIG. 1. Recombinant baculovirus-produced proteins. (A) Stick diagrams of full-length HSV gD and the truncated forms used in this study (produced in recombinant baculovirus-infected cells). Functional regions I to IV as defined by Chiang et al. (7) are shown as shaded regions. The positions of linear epitopes for group II and group VII are indicated. The positions of consensus sites for *N*-glycosylation are marked by balloons. The disulfide bond pattern for the six cysteine residues located in the extracellular domain of gD (36) is shown on the full-length gD stick diagram. (B) Silver-stained SDS-polyacrylamide gel (10%) showing the purified recombinant baculovirus-produced proteins used in this study. Lane 1, gD-1(306t); lane 2, gD-1(285t); lane 3, gD-1(260t); lane 4, gD-1(250t); lane 5, gD-1(240t); lane 6, gD-1(234t); lane 7, gD-1(Δ222-224, 306t). (C) Western blot of the purified proteins shown in panel B probed with MAb 1D3 (group VII).

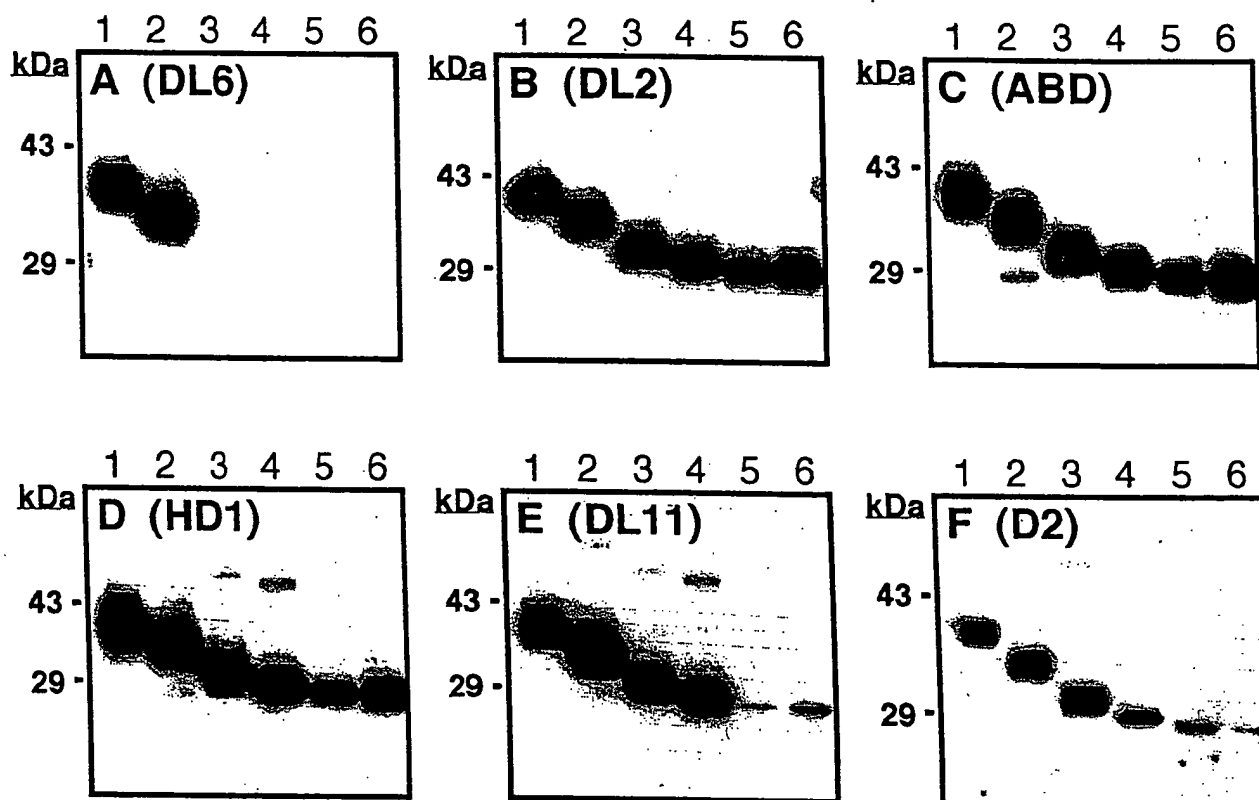


FIG. 2. Antigenic analysis of baculovirus-produced gD truncation mutants. Purified proteins were separated by "native" SDS-PAGE, transferred to nitrocellulose, and probed with gD-specific MAbs. Lane 1, gD-1(306t); lane 2, gD-1(285t); lane 3, gD-1(260t); lane 4, gD-1(250t); lane 5, gD-1(240t); lane 6, gD-1(234t). (A) Blot probed with DL6 (group II MAb). (B) Blot probed with DL2 (group VI MAb). (C) Blot probed with ABD (group III MAb). (D) Blot probed with HD1 (group Ia MAb). (E) Blot probed with DL11 (group Ib MAb). (F) Blot probed with D2 (group Ib MAb).

ative to gD-1(306t) resulted almost exclusively from a faster rate of complex formation (47). In contrast, gD-1(234t) exhibited a faster rate of complex dissociation (k_{off}) with HveA compared to gD-1(306t), suggesting that some gD residues involved in HveA binding had been removed. Here we found

that the binding kinetics and affinities of gD-1(285t), gD-1(260t), and gD-1(250t) for both receptors were quite similar (Table 1). In each case, the higher affinity was due primarily to a faster rate of complex formation (k_{on}). gD-1(240t) exhibited binding kinetics and an affinity similar to gD-1(306t) in its

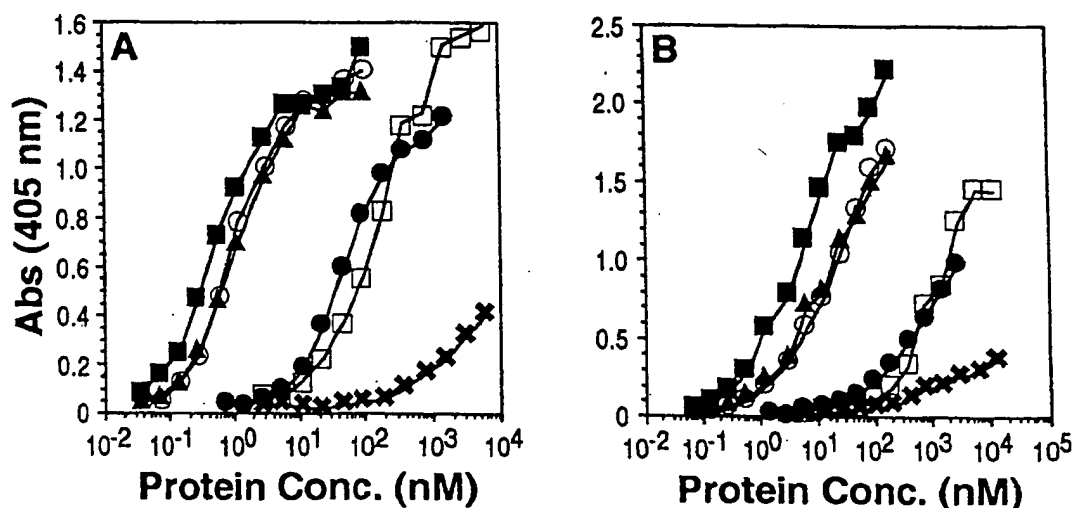


FIG. 3. Analysis of gD truncation mutants for receptor binding by ELISA. The wells of an ELISA plate were coated with an excess of HveA(200t) or HveC(346t) and incubated with increasing concentrations (shown on the x axis) of gD truncation mutants. Bound gD was detected by incubating sample wells with a rabbit antiserum raised against gD (R7), followed by peroxidase-conjugated goat anti-rabbit antibody and then peroxidase substrate. (A) Binding to HveA(200t). (B) Binding to HveC(346t). Symbols: \square , gD-1(306t); \circ , gD-1(285t); Δ , gD-1(260t); \circ , gD-1(250t); \bullet , gD-1(240t); \times , gD-1(234t).

TABLE 1. Kinetic and affinity values for gD-receptor complex formation

Immobilized receptor	gD	k_{on} ($10^3 \text{ s}^{-1} \text{ M}^{-1}$)	k_{off} (10^{-2} s^{-1})	K_D (10^{-6} M) (k_{off}/k_{on})
HveAt	306t ^a	6.1 ± 0.6^b	2.0 ± 0.2^b	3.2 ± 0.6
	285t ^a	300	1.1	0.037
	260t	130	0.75	0.058
	250t	520	0.94	0.018
	240t	18	1.6	0.89
	234t	ND ^c	20 ^d	20 ^d
HveCt	306t ^a	2.6 ± 0.7^b	0.71 ± 0.09^b	2.7 ± 0.2
	285t ^a	190	0.73	0.038
	260t	280	0.71	0.025
	250t	440	0.66	0.015
	240t	25	2.9	1.2
	234t	ND ^c	10 ^d	2 ^d

^a Values for gD-1(306t) (32, 57), gD-1(285t) (32, 47), and the HveA-gD-1(234t) complex (47) were reported previously and are shown here for comparison.

^b Average \pm the standard deviation from at least three separate experiments.

^c ND, not determined.

^d Estimated maximum k_{off} . As a control, this method was also used to calculate a k_{off} for the dissociation of gD-1(306t) from HveA and HveC. The values obtained were $1.5 \times 10^{-2} \text{ s}^{-1}$ and $0.66 \times 10^{-2} \text{ s}^{-1}$, respectively.

^e χ^2 values for the global fits ranged from 0.7 to 4.5.

^f From Scatchard analysis.

interaction with HveA, consistent with its similar binding properties as determined by ELISA. Although gD-1(240t) exhibited an overall affinity for HveC similar to that of gD-1(306t), the k_{on} was 10-fold faster and the k_{off} was 4-fold faster than gD-1(306t). Thus, in contrast to the ELISA results, the optical biosensor enabled us to distinguish the binding of gD-1(306t) versus gD-1(240t) to HveC. As observed previously (47), gD-1(234t) bound to HveAt, but the data failed to fit a 1:1 Langmuir binding model. A similar result was obtained when gD-1(234t) binding to HveC was examined. Because of this, the binding kinetics for gD-1(234t) could not be analyzed by using the global fitting routine of the instrument software. Instead, maximum k_{off} values were estimated by plotting $\ln(R_0/R_n)$ ver-

sus time for the initial part of the dissociation phase. For both HveA and HveC, the maximum k_{off} for gD-1(234t) was approximately 10-fold faster than for gD-1(306t). Finally, equilibrium dissociation constants (K_D) for gD-1(234t) binding to HveA and HveC were calculated from data collected under conditions of binding equilibrium by using Scatchard analysis. For gD-1(234t) binding to HveA this calculation yielded a K_D approximately sixfold lower than gD-1(306t), while for binding to HveC the K_D was nearly identical to that of gD-1(306t).

Blocking of virus entry by soluble forms of gD. Soluble gD blocks HSV entry into susceptible cells, presumably by binding to and occupying cell surface receptors (28, 45). We tested the abilities of truncated gD to block infection of HeLa and Vero cells (Fig. 4A and B). gD-1(285t), gD-1(260t), and gD-1(250t) blocked HSV infection at similar concentrations (50% inhibition occurred between 1 and 10 nM) and were more potent than gD-1(306t) (50% inhibition occurred between 100 and 200 nM). These results were consistent with the ELISA and biosensor data showing that gD-1(285t), gD-1(260t), and gD-1(250t) bound to both HveA and HveC with greatly increased affinities relative to gD-1(306t). Similarly, the weak ability of gD-1(234t) to block HSV infection is consistent with its diminished capacity to bind HveA or HveC relative to gD-1(306t) (at least by ELISA). Surprisingly, gD-1(240t) blocked HSV infection much less effectively than gD-1(306t) (50% inhibition occurred at approximately 5 μM). This result was unexpected because both the ELISA and biosensor data indicated that this protein bound to both receptor molecules with an affinity similar to gD-1(306t). We have repeated these experiments several times with similar results. These data suggest that gD-1(240t) may lack a portion of a gD functional domain.

Antigenic structure of 3-amino-acid deletion mutants. In order to characterize further the region of gD encompassing functional region III, as well as the residues involved in group Ib MAbs binding, we used site-directed mutagenesis to generate a series of plasmids encoding full-length gD with nonoverlapping 3-amino-acid deletions spanning amino acid residues 222 through 254 (Fig. 5). COS cells were transfected separately with plasmids expressing each of the gD mutants. With the

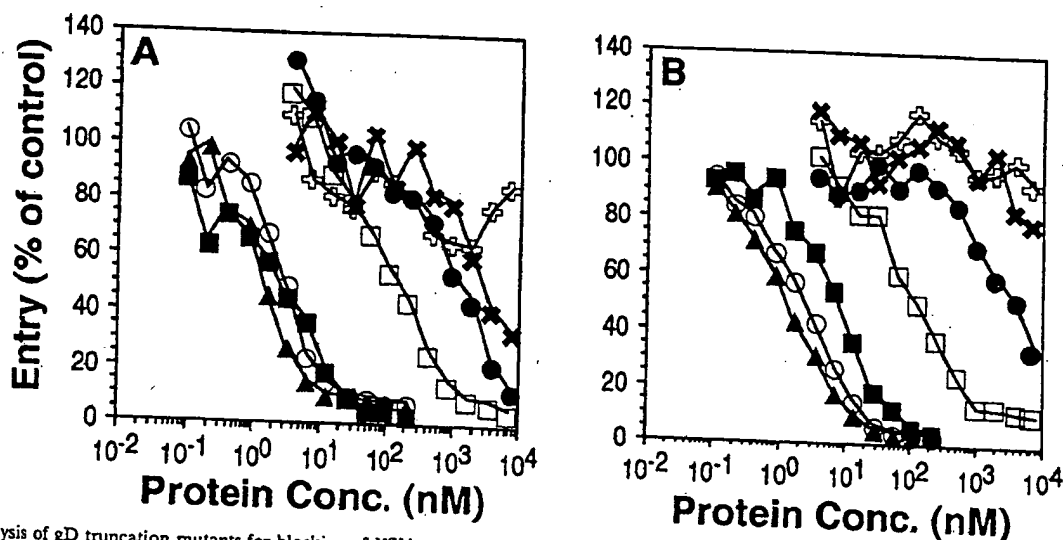


FIG. 4. Analysis of gD truncation mutants for blocking of HSV entry. Cells in 96-well tissue culture plates were incubated with increasing concentrations (shown on the x axis) of various forms of gD prior to infection with HSV-1(KOS) carrying a β -galactosidase reporter gene (*hrR3*). At 5 h postinfection, cells were lysed and assayed for β -galactosidase activity. (A) HeLa cells. (B) Vero cells. Symbols: \square , gD-1(306t); \circ , gD-1(285t); \triangle , gD-1(260t); ∇ , gD-1(250t); \bullet , gD-1(240t); \diamond , gD-1(234t); \oplus , BSA.

no Acid Sequence	Deletion	Complementation
255		
VYSLKIAGWEGPKAPYTTSTLLPF	NONE	100%
VYSLKIAGWEGPKAPYTTSTLLPF	222-224	2
VYSLKIAGWEGPKAPYTTSTLLPF	225-227	0
VYSLKIAGWEGPKAPYTTSTLLPF	228-230	1
VYSLKIAGWEGPKAPYTTSTLLPF	231-233	1
VYSLKIAGWEGPKAPYTTSTLLPF	234-236	0
VYSLKIAGWEGPKAPYTTSTLLPF	237-239	2
VYSLKIAGWEGPKAPYTTSTLLPF	240-242	ND
VYSLKIAGWEGPKAPYTTSTLLPF	243-245	0
VYSLKIAGWEGPKAPYTTSTLLPF	246-248	0
VYSLKIAGWEGPKAPYTTSTLLPF	249-251	1
VYSLKIAGWEGPKAPYTTSTLLPF	252-254	15

ementation analysis of 3-amino-acid deletion mutants of edicted amino acid sequence for residues 220 to 255 of HSV-1 vn at the top left (numbering is based on the assignment of the lysine at the N terminus of the signal-peptidase-product, which is the 26th residue of the primary translation product).) has two amino acid changes relative to gD from the KOS n of the protein, V→L at residue 233 and A→P at residue 246. e sequence, the corresponding sequences of the 3-amino-acid re shown. The level of complementation of a gD-null virus is onstruct in the column at the right and is expressed as a t achieved with the wild-type construct (the mutant lacking 2 was never detected in transfected cells [see text]).

re Δ240-242 deletion mutant, all of the mutated transported to the surface of transfected cells, as immunoperoxidase staining (data not shown). Cy- acts were then prepared from COS cells 40 h tion. As controls, plasmids pRE4, which ex- type gD-1, and pWW17, which expresses the tion mutant (12), were included. To quantitate pression, equal volumes of each extract were d on a denaturing polyacrylamide gel, followed nitrocellulose and probing with MAb DL6 (26). result, the volumes of extract loaded on subse- e normalized so as to give approximately equal L6 (Fig. 6A). No protein could be detected for 242, even after repetition of the mutagenesis, so l from further analysis. Each extract was then d on a native polyacrylamide gel with no comb, trocellulose were cut from the resulting Western d with various MAbs (Fig. 6B to D). MAbs HD1 ABD (panel C), which recognize discontinuous itigenic sites Ia and III, respectively (41-43), of the mutant proteins. The binding of DL11 eliminated by deletion of residues 222 to 224, 1 228 to 230 (lanes 1 to 3, respectively), sugges- es 222 to 230 contribute to antigenic site Ib. leletion mutants in a complementation assay. as tested for its ability to complement the pro- ctious F-gDβ virus in COS cells by using quan- id DNA that result in similar numbers of gD- ls. F-gDβ lacks a gD gene and produces ; only when functional gD protein is provided in h the exception of Δ252-254, none of the mu- to complement F-gDβ, as found previously for mutant (42) (Fig. 5). To address the possibility mplementation was due to failure of the mutated incorporated into virions, extracellular comple- virus was centrifuged through a 10% sucrose 00 × g for 2 h at 4°C. The pellet was solubilized DS-PAGE sample buffer, electrophoresed on a amide gel, Western blotted, and probed with

polyclonal antibodies against gD and gB (Fig. 7). Although each of the mutant proteins was detected in virions, the Δ225-227 and Δ243-245 proteins were incorporated inefficiently and may explain their complementation-negative phenotype. Taken together, these results suggest that a region encompassing at least residues 222 to 251 is required for gD function.

Expression of gD-1(Δ222-224) and gD-1(Δ231-233) as soluble forms in the baculovirus system. In order to examine the receptor-binding properties of a subset of the 3-amino-acid gD deletion mutants, we constructed two baculovirus recombinants expressing gD-1 truncated after residue 306 and lacking residues 222 to 224 (DL11 negative) or residues 231 to 233 (DL11 positive). While both proteins were detected in recombinant baculovirus-infected insect cells, only the Δ222-224 protein [gD-1(Δ222-224, 306t)] was secreted. The baculovirus-produced gD-1(Δ222-224, 306t) was purified by nickel-agarose chromatography (Fig. 1), and its reactivity with a panel of MAbs was analyzed by native Western blot (Fig. 8). As antic-

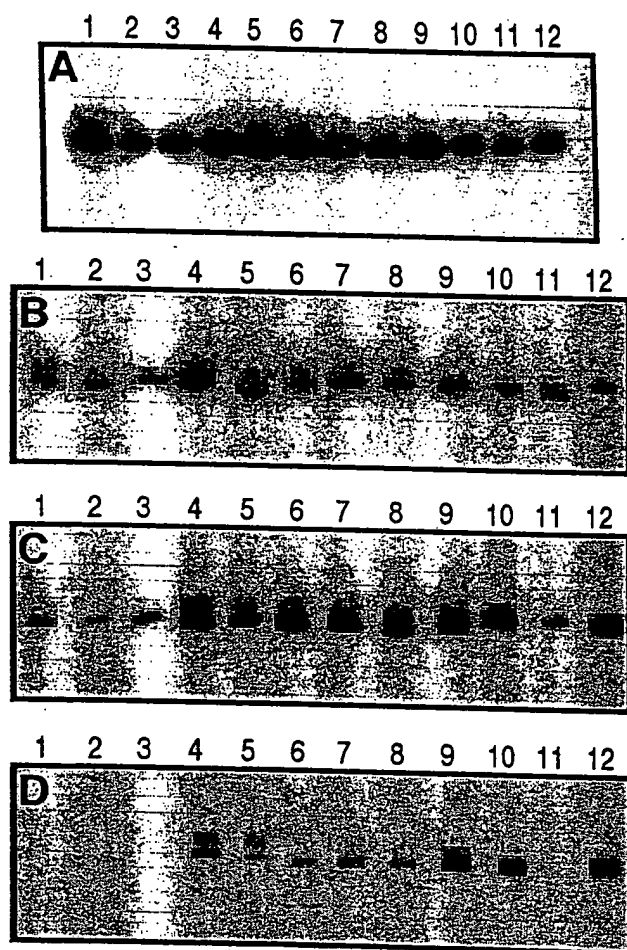


FIG. 6. Antigenic analysis of HSV-1 gD 3-amino-acid deletion mutants. Replicate cultures of COS cells were transfected separately with plasmid constructs expressing wild-type gD, gD lacking residues 234 to 244, and each of the 3-amino-acid deletion mutants. Detergent extracts were prepared from transfected cells after 40 h, subjected to SDS-PAGE, and transferred to nitrocellulose (Western blot). Blots were then probed with MAbs against HSV gD. (A) Blot probed with DL6. (B) Blot probed with ABD. (C) Blot probed with HD1. (D) Blot probed with DL11. Lane 1, gD-1(Δ222-224); lane 2, gD-1(Δ225-227); lane 3, gD-1(Δ228-230); lane 4, gD-1(Δ231-233); lane 5, gD-1(Δ234-236); lane 6, gD-1(Δ237-239); lane 7, gD-1(Δ243-245); lane 8, gD-1(Δ246-248); lane 9, gD-1(Δ249-251); lane 10, gD-1(Δ252-254); lane 11, gD-1(Δ234-244); lane 12, wild-type gD-1.

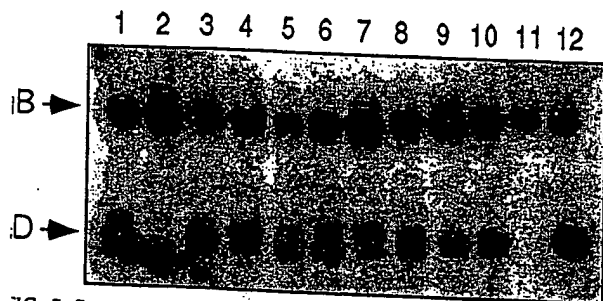


FIG. 7. Detection of gD in F-gD virus complemented with the 3-amino-acid deletion mutants. Extracellular, F-gD virus which had been complemented separately with each of the 3-amino-acid deletion mutants was prepared as described in the text and analyzed by SDS-PAGE followed by Western blotting. The resulting blot was probed with a mixture of two polyclonal antibodies, R7 (raised against gD) and R69 (raised against gB). Lane 1, virus complemented with gD-1(Δ 222-224); lane 2, virus complemented with gD-1(Δ 225-227); lane 3, virus complemented with gD-1(Δ 228-230); lane 4, virus complemented with gD-1(Δ 231-233); lane 5, virus complemented with gD-1(Δ 234-236); lane 6, virus complemented with gD-1(Δ 237-239); lane 7, virus complemented with gD-1(Δ 243-245); lane 8, virus complemented with gD-1(Δ 246-248); lane 9, virus complemented with gD-1(Δ 249-251); lane 10, virus complemented with gD-1(Δ 252-254); lane 11, cells transfected with salmon sperm DNA; lane 12, virus complemented with wild-type gD-1.

ected from the data shown in Fig. 5, this mutant form of gD reacted with MAbs ABD and HD1. The deletion mutant also reacted as well as gD-1(306t) with MAbs DL6 and DL2, further validating its structural integrity. In contrast, gD-1(Δ 222-224, 306t) failed to react with either of two group Ib MAbs tested, DL11 and D2. These results were consistent with the antigenic properties of the full-length form of this protein expressed in mammalian cells (see Fig. 6). gD-1(Δ 222-224, 306t) binds poorly to HveA and HveC and fails to block HSV infection. To assess the receptor-binding properties of gD-1(Δ 222-224, 306t), we examined its ability to bind to HveA(200t) and to HveC(346t) by ELISA. As shown in Fig. 9A, the binding of gD-1(Δ 222-224, 306t) to HveA(200t) was barely detectable, even at concentrations as high as 6 μ M. Binding to HveC(346t) was diminished by ca. 10-fold relative to that of gD-1(306t). Consistent with its reduced binding to both HveA and HveC, gD-1(Δ 222-224, 306t) failed to block infection of HeLa or Vero cells (Fig. 10). The failure of gD-1(Δ 222-224, 306t) to bind well to either of two known HSV receptors most likely explains the inability of the full-length form of this protein to block virus infection or to complement infectivity of a gD-null virus.

DISCUSSION

During the past decade, numerous studies have examined the structure of HSV gD relates to its function. Some

studies focused on a discontinuous antigenic site which was bound by several type-common, complement-independent neutralizing MAbs (antigenic site I). On the basis of additional characteristics, group I MAbs were subdivided into subgroups Ia and Ib (41). For example, the group Ia MAb, HD1, binds to gD truncated at amino acid residue 233, whereas the group Ib antibodies, such as DL11, do not. Separate studies demonstrated the involvement of residues upstream of 233 in antigenic site Ib as well (39, 43). Single-amino-acid changes were identified which allowed gD to function during virus replication while conferring resistance to neutralization by certain group I antibodies. Specifically, two group Ib antibodies failed to neutralize a virus expressing gD with a Ser-to-Asn change at residue 140, and a third group Ib antibody failed to neutralize a virus expressing gD with a Gln-to-Leu change at residue 132 (Fig. 11).

Evidence of an overlap between antigenic site Ib and a putative functional region of gD was provided by Muggeridge et al. (42), who examined seven gD mutants containing N-terminal, internal, or C-terminal amino acid deletions for their ability to complement a gD-null virus. gD lacking residues 234 to 244 was expressed on the surface of transfected cells and, although not globally altered in structure, failed to rescue the infectivity of a gD-null virus. Interestingly, this mutant protein failed to react with DL11, suggesting that antigenic site Ib, as well as a functional region of gD, was disrupted by this 11-amino-acid deletion. In a similar study, Feenstra et al. (17) found that deletion of residues 231 to 235 resulted in a protein which also failed to complement a gD-null virus but retained reactivity with DL11. More recently, Nicola et al. (44) showed that DL11 blocked the interaction of soluble HveA with gD or with HSV virions, and Krummenacher et al. (31) showed that DL11 blocked the interaction of soluble HveC with HSV virions. Finally, gD truncated at residue 234 was bound by DL11 weakly and bound HveA with a markedly lower affinity (K_D) than molecules truncated at residue 275, 285, or 306 (47).

Linker-scanning mutational analysis of HSV gD (7) identified four distinct functional regions within the gD primary structure wherein linker insertions did not cause global structural alterations but greatly diminished or eliminated the protein's ability to complement the infectivity of a gD-null virus (shaded areas designated I through IV in Fig. 11). This study also revealed a relationship between antigenic site Ib and regions II and III. First, linker insertions within region II abolished or greatly diminished binding by DL11. Second, region II (residues 125 to 161) encompasses residues previously shown to affect the binding of group Ib antibodies (residues 132 and 140; see Fig. 11). Third, region III (residues 225 to 246) includes residues required for group Ib antibody binding. These observations suggest that regions II and III may be closely

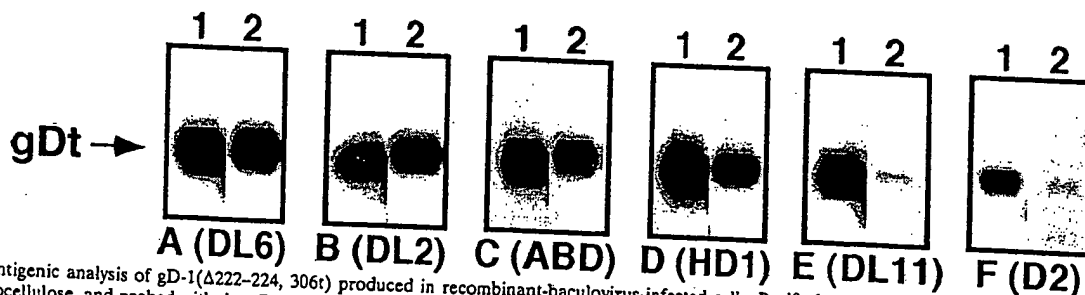
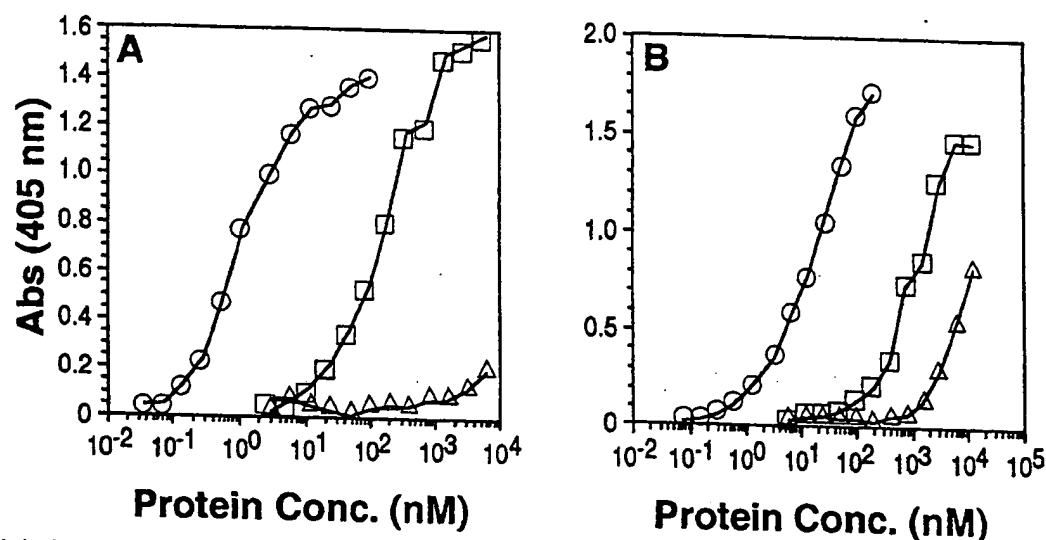


Fig. 8. Antigenic analysis of gD-1(Δ 222-224, 306t) produced in recombinant-baculovirus-infected cells. Purified proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with the gD-specific MAbs indicated below each panel. Lane 1, gD-1(306t); lane 2, gD-1(Δ 222-224, 306t). The gD-1(306t) bands in each panel correspond to those shown in Fig. 2A to F.



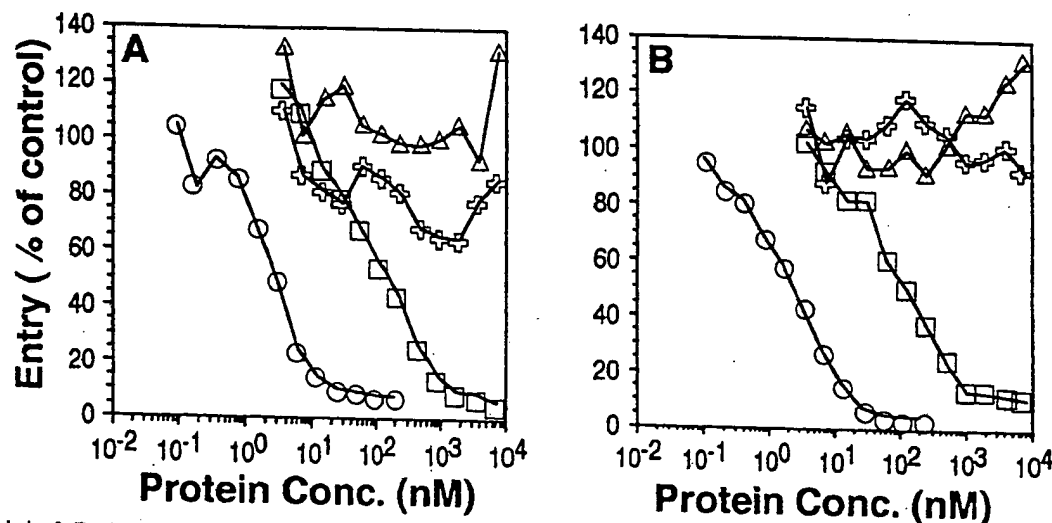
1. Analysis of gD-1(Δ222-224, 306t) for receptor binding by ELISA. The wells of an ELISA plate were coated with an excess of HveA(200t) or HveC(346t) and incubated with increasing concentrations (shown on the x axis) of gD-1(306t), gD-1(250t), or gD-1(Δ222-224, 306t). Bound gD was detected by incubating sample with a rabbit antiserum raised against gD (R7), followed by peroxidase-conjugated goat anti-rabbit antibody and then peroxidase substrate. (A) Binding to HveA(200t). (B) Binding to HveC(346t). Symbols: □, gD-1(306t); ○, gD-1(250t); △, gD-1(Δ222-224, 306t).

located within the folded structure of gD and may, together, form a functional (receptor-binding) domain.

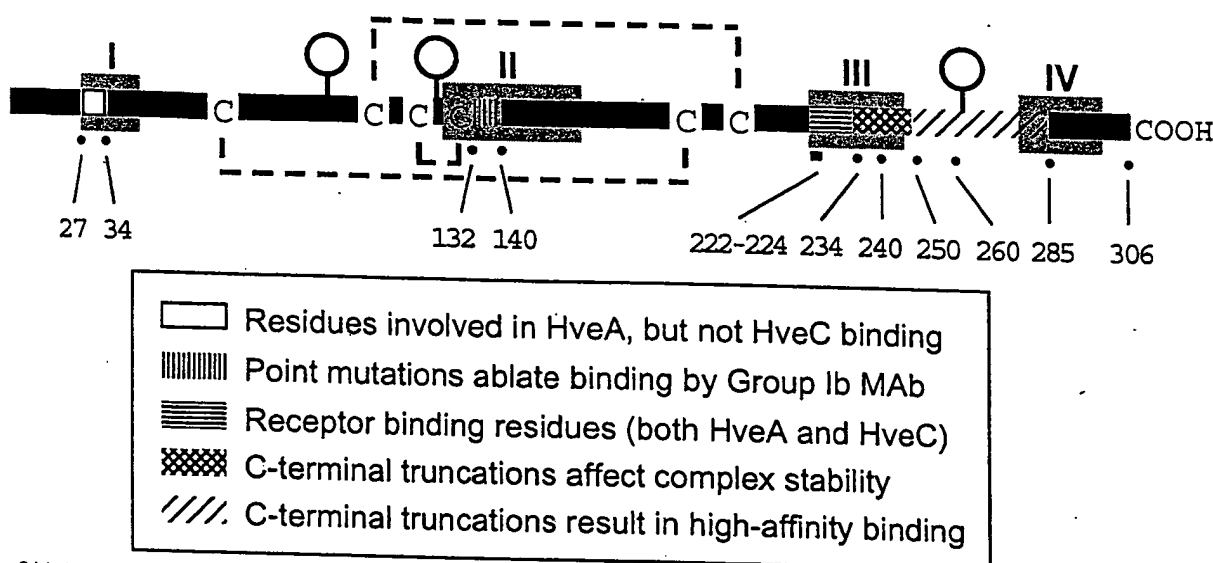
In the present study we addressed the contribution of gD residues between 222 and 275 to the formation of both anti-gD as well as a receptor-binding domain. We constructed three baculovirus recombinants expressing gD truncations between residues 240, 250, and 260 and analyzed them along with previously described gD truncations (after residues 234, 240, and 306) for antigenic structure, receptor binding, and blocking activity. All of the truncated proteins were recognized by several MAbs recognizing discontinuous epitopes. In particular, reactivity with DL11 was not exhibited by all of the truncated mutants. Although gD-1(250t) reacted strongly with gD-1(240t) and gD-1(234t) had significantly diminished

reactivity. Thus, we conclude that the C-terminal limit for full DL11 binding occurs between residues 240 and 250.

Analysis of the C-terminal truncation mutants for receptor binding revealed a pattern somewhat similar to that seen for DL11 binding. Using the activity of gD-1(306t) as a reference point, gD-1(285t), gD-1(260t), and gD-1(250t) exhibited enhanced binding to both HveA and HveC (Fig. 11), a property previously demonstrated for gD-1(285t) and gD-1(275t) (31, 47). The higher affinity of gD-1(285t) and gD-1(275t) for HveA was shown by optical biosensor studies to result from a faster k_{on} . Here we found that gD-1(260t) and gD-1(250t) bound to both HveA and HveC with kinetics and K_D values very similar to those of gD-1(285t). The calculated K_D values for the interactions of gD-1(240t) with HveA and HveC were quite similar



2. Analysis of gD-1(Δ222-224, 306t) for blocking of HSV entry. Cells in 96-well tissue culture plates were incubated with increasing concentrations (shown on the x axis) of gD-1(306t), gD-1(250t), or gD-1(Δ222-224, 306t) prior to infection with HSV-1(KOS) carrying a β -galactosidase reporter gene (*hrR3*). At 5 h post-infection, cells were lysed and assayed for β -galactosidase activity. (A) HeLa cells. (B) Vero cells. Symbols: □, gD-1(306t); ○, gD-1(250t); △, gD-1(Δ222-224, 306t); +, serum albumin.



Stick diagram of HSV gD showing features relevant to this study. The amino- and carboxy-terminal ends of the gD ectodomain are indicated by H₂N and COOH, respectively. Cysteine residues are denoted by C, and the disulfide bond pattern (36) is indicated by dashed lines. Functional regions I to IV as defined by Chiang et al. (7) are shaded in gray. The positions of individual residues relevant to the present study are marked by a dot and labeled with the residue number.

for gD-1(306t). Interestingly, the k_{on} and k_{off} values for the interaction of gD-1(240t) with HveC were typically faster than those obtained for gD-1(306t). From experiments it is clear that the loss of high-affinity receptor binding by gD is first evident in gD-1(240t), the same point at which full DL11 reactivity is lost. The truncation, gD-1(234t), exhibited an approximately 10-fold k_{off} for both HveA and HveC relative to gD-1(306t), due to deletion of gD residues which stabilize the structure (Fig. 11).

Ability of soluble gD to bind receptor molecules should directly correspond to its ability to block HSV infection of cells bearing those receptors. In the case of both HeLa and Vero cells, the blocking activities of all but one of the proteins examined matched their receptor-binding properties as seen by competition experiments. Interestingly, gD-1(240t), which bound both HveA and HveC with a K_D very similar to gD-1(306t), was much less effective in blocking HSV infection than gD-1(306t). Perhaps membrane-bound forms of HveA and HveC recognize gD differently than the truncated forms used in the competition biosensor studies. This result might also suggest that there is yet another receptor in HeLa and Vero cells to which gD-1(240t) binds with lower affinity than gD-1(306t). This explanation seems unlikely, at least for HeLa cells, since Chiang et al. (8) showed that a MAb to HIGR (HveC) effectively blocks HSV infection of this cell line. Alternatively, the difference in blocking ability between gD-1(306t) and gD-1(240t) may reflect the different rates of gD-HveC complex formation and/or dissociation observed in the optical biosensor studies discussed above.

The observation that gD-1(234t) bound HveA, albeit in a noncompetitive manner, indicated that at least some receptor-binding sites were present upstream of 234. To extend our analysis of the group Ib epitope and functional region III, we generated a series of plasmid constructs expressing full-length HSV-1 gD with sequential, nonoverlapping, 3-amino-acid deletions extending from residue 222 through residue 254. The altered gD proteins were expressed in transiently transfected cells and were first assayed for function by using a complementation assay. Only 3-amino-acid gD deletion mutants (Δ 252-254) com-

plemented the infectivity of a gD-null virus, confirming the conclusions of Chiang et al. (7). All forms of gD retained the folded structure necessary for reactivity with group III and group Ia MAbs, and each was detected on the surface of transfected cells. However, gD lacking residues 222 to 224, 225 to 227, or 228 to 230 failed to react with DL11, indicating that even small deletions in this region of gD disrupt antigenic site Ib. Earlier results, examined in connection with data presented here, suggest that gD residues 222 through 230 are critical for proper formation of the DL11 epitope, whereas residues between positions 231 and 250 may be important for proper presentation of the DL11 epitope but are not directly involved in antibody binding.

To examine the receptor-binding properties of some of the 3-amino-acid deletion mutants, we cloned and expressed two of these proteins (Δ 222-224 and Δ 231-233) as truncated forms in the baculovirus system. Although both of these molecules were detected in recombinant-baculovirus-infected cells, only gD-1(Δ 222-224, 306t) could be purified from the culture medium. gD-1(Δ 222-224, 306t) reacted with several MAbs (but not DL11, as expected), bound weakly to HveA and HveC, and failed to block HSV infection of mammalian cells. This result showed that gD-1(Δ 222-224) is nonfunctional due, at least in part, to its greatly diminished binding to cellular receptors. Once again, these data support the concept of an overlap between a receptor-binding domain of gD and the DL11 epitope.

Antibodies directed to viral proteins can neutralize virus infectivity by binding to and occupying the site on a virion protein which interacts with a cellular receptor during virus attachment and entry. This mechanism of neutralization by certain MAbs has been demonstrated for several different viruses, including influenza virus (3, 51). Whether DL11 (and other group Ib MAbs) neutralizes HSV infectivity by occupying part of its receptor binding domain has yet to be conclusively demonstrated. The data presented here and in previous publications are clearly consistent with this interpretation, although it is formally possible that the receptor-binding site on gD is spatially distinct from the group Ib antigenic site. To address this and other questions, we are currently attempting

to determine the crystal structure of gD alone, as well as gD complexed with each of its two known receptors or complexed with the DL11 MAb.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants AI-18289 to G.H.C. and R.J.E. from the National Institute of Allergy and Infectious Diseases and grants NS-30606 and NS-36731 to R.J.E. and G.H.C. from the National Institute of Neurologic Diseases and Stroke. C.K. was supported by a fellowship (823A-053464) from the Swiss National Science Foundation.

REFERENCES

- Aoki, J., S. Koike, H. Asou, I. Ise, H. Suwa, T. Tanaka, M. Miyasaka, and A. Nomoto. 1997. Mouse homolog of poliovirus receptor-related gene 2 product, mPRR2, mediates homophilic cell aggregation. *Exp. Cell Res.* 235:374-384.
- Biacore, Inc. 1997. BIAevaluation software handbook, version 3.0. Biacore, Inc., Uppsala, Sweden.
- Bizebard, T., B. Gigant, P. Rigolet, B. Rasmussen, O. Diat, P. Bosecke, S. Wharton, J. Skehel, and M. Knossow. 1995. Structure of influenza virus haemagglutinin complexed with a neutralizing antibody. *Nature* 376:92-94.
- Cal, W., S. Person, C. DebRoy, and B. Gu. 1988. Functional regions and structural features of the gB glycoprotein of herpes simplex virus type 1. *J. Mol. Biol.* 201:575-588.
- Carswell, S., J. Resnick, and J. C. Alwine. 1986. Construction and characterization of CV-1P cell lines which constitutively express the simian virus 40 agnoprotein: alteration of plaquing phenotype of viral agnogene mutants. *J. Virol.* 60:415-422.
- Chen, E. U., and P. H. Seeberg. 1985. Supercil sequencing: a fast and simple method for sequencing plasmid DNA. *DNA* 4:165-170.
- Chiang, H.-Y., G. H. Cohen, and R. J. Eisenberg. 1994. Identification of functional regions of herpes simplex virus glycoprotein gD by using linker-insertion mutagenesis. *J. Virol.* 68:2529-2543.
- Cocchi, F., M. Lopez, L. Menotti, M. Aoubala, P. Dubreuil, and G. Campadelli-Fiume. 1998. The V domain of herpesvirus Ig-like receptor (HlgR) contains a major functional region in herpes simplex virus-1 entry into cells and interacts physically with the viral glycoprotein D. *Proc. Natl. Acad. Sci. USA* 95:15700-15705.
- Cocchi, F., L. Menotti, P. Mirandola, M. Lopez, and G. Campadelli-Fiume. 1998. The ectodomain of a novel member of the immunoglobulin subfamily related to the poliovirus receptor has the attribute of a bona fide receptor for herpes simplex virus types 1 and 2 in human cells. *J. Virol.* 72:9992-10002.
- Cohen, G. H., B. Dietzschold, M. Ponce de Leon, D. Long, E. Golub, A. Varrichio, L. Pereira, and R. J. Eisenberg. 1984. Localization and synthesis of an antigenic determinant of herpes simplex virus glycoprotein D that stimulates production of neutralizing antibody. *J. Virol.* 49:102-108.
- Cohen, G. H., V. J. Isola, J. Kuhns, P. W. Berman, and R. J. Eisenberg. 1986. Localization of discontinuous epitopes of herpes simplex virus glycoprotein D: use of a nondenaturing ("native") gel system of polyacrylamide gel electrophoresis coupled with Western blotting. *J. Virol.* 60:157-166.
- Cohen, G. H., W. C. Wilcox, D. L. Sodora, D. Long, J. Z. Levin, and R. J. Eisenberg. 1988. Expression of herpes simplex virus type 1 glycoprotein D deletion mutants in mammalian cells. *J. Virol.* 62:1932-1940.
- Dietzschold, B., R. J. Eisenberg, M. Ponce de Leon, E. Golub, F. Hudecz, A. Varrichio, and G. H. Cohen. 1984. Fine structure analysis of type-specific and type-common antigenic sites of herpes simplex virus glycoprotein D. *J. Virol.* 52:431-435.
- Eberlé, F., P. Dubreuil, M.-G. Mattei, E. Devillard, and M. Lopez. 1995. The human PRR2 gene, related to the poliovirus receptor gene (PVR), is the true homolog of the murine MPH gene. *Gene* 159:267-272.
- Eisenberg, R. J., D. Long, M. Ponce de Leon, J. T. Matthews, P. G. Spear, M. G. Gibson, L. A. Lasky, P. Berman, E. Golub, and G. H. Cohen. 1985. Localization of epitopes of herpes simplex virus type 1 glycoprotein D. *J. Virol.* 53:634-644.
- Eisenberg, R. J., M. Ponce de Leon, H. M. Friedman, L. F. Fries, M. M. Frank, J. C. Hastings, and G. H. Cohen. 1987. Complement component C3b binds directly to purified glycoprotein C of herpes simplex virus types 1 and 2. *Microb. Pathog.* 3:423-435.
- Feenstra, V., M. Hodaie, and D. C. Johnson. 1990. Deletions in herpes simplex virus glycoprotein D define nonessential and essential domains. *J. Virol.* 64:2096-2102.
- Friedman, H. M., G. H. Cohen, R. J. Eisenberg, C. A. Seidel, and D. B. Cines. 1984. Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. *Nature (London)* 309:633-635.
- Geraghty, R. J., C. Krummenacher, G. H. Cohen, R. J. Eisenberg, and P. G. Spear. 1998. Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* 280:1618-1620.
- Goldstein, D. J., and S. K. Weller. 1988. Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 *lacZ* insertion mutant. *J. Virol.* 62:196-205.
- Herold, B. C., R. J. Visalli, N. Sumarski, C. Brandt, and P. G. Spear. 1994. Glycoprotein C-independent binding of herpes simplex virus to cells requires cell surface heparan sulfate and glycoprotein B. *J. Gen. Virol.* 75:1211-1222.
- Herold, B. C., D. WuDunn, N. Soltys, and P. G. Spear. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J. Virol.* 65:1090-1098.
- Highlander, S. L., S. L. Sutherland, P. J. Gage, D. C. Johnson, M. Levine, and J. C. Glorioso. 1987. Neutralizing monoclonal antibodies specific for herpes simplex virus glycoprotein D inhibit virus penetration. *J. Virol.* 61:3356-3364.
- Holland, T. C., S. D. Martin, M. Levine, and J. Glorioso. 1983. Antigenic variants of herpes simplex virus selected with glycoprotein-specific monoclonal antibodies. *J. Virol.* 45:672-682.
- Huang, A., and R. Wagner. 1964. Penetration of herpes simplex virus into human epidermoid cells. *Proc. Soc. Exp. Biol. Med.* 116:863-869.
- Isola, V. J., R. J. Eisenberg, G. R. Siebert, C. J. Heilman, W. C. Wilcox, and G. H. Cohen. 1989. Fine mapping of antigenic site II of herpes simplex virus glycoprotein D. *J. Virol.* 63:2325-2334.
- Johnson, D. C., R. L. Burke, and T. Gregory. 1990. Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. *J. Virol.* 64:2569-2576.
- Johnson, D. C., T. Gregory, and R. L. Burke. 1989. Inhibition of HSV entry into cells by purified HSV glycoprotein D and characterization of cell surface receptors for HSV, p. 237. 14th International Herpesvirus Workshop, Nyborg Strand, Denmark.
- Karlsson, R., A. Michaelsson, and A. Mattsson. 1991. Kinetic analysis of monoclonal antibody-antigen interactions with a new biosensor based analytical system. *J. Immunol. Methods* 145:229-240.
- Kousoulas, K. G., P. E. Pellett, L. Pereira, and B. Roizman. 1984. Mutations affecting conformation or sequence of neutralizing epitopes identified by reactivity of viable plaques segregate from *syn* and *ts* domains of HSV-1(F) gB gene. *Virology* 135:379-394.
- Krummenacher, C., A. V. Nicola, J. C. Whitbeck, H. Lou, W. Hou, J. D. Lambiris, R. J. Geraghty, P. G. Spear, G. H. Cohen, and R. J. Eisenberg. 1998. Herpes simplex virus glycoprotein D can bind to poliovirus receptor-related protein 1 or herpesvirus entry mediator, two structurally unrelated mediators of virus entry. *J. Virol.* 72:7064-7074.
- Krummenacher, C., A. H. Rux, J. C. Whitbeck, M. Ponce-de-Leon, H. Lou, I. Baribaud, W. Hou, C. Zou, R. J. Geraghty, P. G. Spear, R. J. Eisenberg, and G. H. Cohen. 1999. The first immunoglobulin-like domain of HveC is sufficient to bind herpes simplex virus gD with full affinity, while the third domain is involved in oligomerization of HveC. *J. Virol.* 73:8127-8137.
- Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* 82:488-492.
- Kunkel, T. A., J. D. Roberts, and R. A. Zakour. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154:367-382.
- Ligas, M. W., and D. C. Johnson. 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β -galactosidase sequences binds to but is unable to penetrate into cells. *J. Virol.* 62:1486-1494.
- Long, D., W. C. Wilcox, W. R. Abrams, G. H. Cohen, and R. J. Eisenberg. 1992. Disulfide bond structure of glycoprotein D of herpes simplex virus types 1 and 2. *J. Virol.* 66:6668-6685.
- Lopez, M., F. Eberlé, M.-G. Mattei, J. Gabert, F. Birg, F. Bardin, C. Maroc, and P. Dubreuil. 1995. Complementary DNA characterization and chromosomal localization of a human gene related to the poliovirus receptor-encoding gene. *Gene* 155:261-265.
- Mauri, D. N., R. Ebner, K. D. Kochel, R. I. Montgomery, T. C. Cheung, G.-L. Yu, M. Murphy, R. J. Eisenberg, G. H. Cohen, P. G. Spear, and C. F. Ware. 1998. LIGHT, a new member of the TNF superfamily, and lymphotoxin (LT) α are ligands for herpesvirus entry mediator (HVEM). *Immunity* 8:21-30.
- Minson, A. C., T. C. Hodgman, P. Digard, D. C. Hancock, S. E. Bell, and E. A. Buckmaster. 1986. An analysis of the biological properties of monoclonal antibodies against glycoprotein D of herpes simplex virus and identification of amino acid substitutions that confer resistance to neutralization. *J. Gen. Virol.* 67:1001-1013.
- Montgomery, R. I., M. S. Warner, B. J. Lum, and P. G. Spear. 1996. Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* 87:427-436.
- Muggeridge, M. I., V. J. Isola, R. A. Byrn, T. J. Tucker, A. C. Minson, J. C. Glorioso, G. H. Cohen, and R. J. Eisenberg. 1988. Antigenic analysis of a major neutralization site of herpes simplex virus glycoprotein D, using deletion mutants and monoclonal antibody-resistant mutants. *J. Virol.* 62:3274-3280.
- Muggeridge, M. I., W. C. Wilcox, G. H. Cohen, and R. J. Eisenberg. 1990. Identification of a site on herpes simplex virus type 1 gD that is essential for infectivity. *J. Virol.* 64:3617-3626.
- Muggeridge, M. I., T.-T. Wu, D. C. Johnson, J. C. Glorioso, R. J. Eisenberg, and G. H. Cohen. 1990. Antigenic and functional analysis of a neutralization

- site of HSV-1 glycoprotein D. *Virology* 174:375-387.
44. Nicola, A. V., M. Ponce de Leon, R. Xu, W. Hou, J. C. Whitbeck, C. Krummenacher, R. I. Montgomery, P. G. Spear, R. J. Eisenberg, and G. H. Cohen. 1998. Monoclonal antibodies to distinct sites on the herpes simplex virus (HSV) glycoprotein D block HSV binding to HVEM. *J. Virol.* 72:3595-3601.
 45. Nicola, A. V., S. H. Willis, N. N. Naidoo, R. J. Eisenberg, and G. H. Cohen. 1996. Structure-function analysis of soluble forms of herpes simplex virus glycoprotein D. *J. Virol.* 70:3815-3822.
 46. Pereira, L., T. Klassen, and J. R. Baringer. 1980. Type-common and type-specific monoclonal antibody to herpes simplex virus type 1. *Infect. Immun.* 29:724-732.
 47. Rux, A. H., S. H. Willis, A. V. Nicola, W. Hou, C. Peng, H. Lou, G. H. Cohen, and R. J. Eisenberg. 1998. Functional region IV of glycoprotein D from herpes simplex virus modulates glycoprotein binding to the herpes virus entry mediator. *J. Virol.* 72:7091-7098.
 48. Seigneurin, J. M., C. Desgranges, D. Seigneurin, J. Paire, J. C. Renversez, B. Jacquemont, and C. Micouin. 1983. Herpes simplex virus glycoprotein D: human monoclonal antibody produced by bone marrow cell line. *Science* 221:173-175.
 49. Sisk, W. P., J. D. Bradley, R. J. Leipold, A. M. Stoltzfus, M. Ponce de Leon, M. Hilf, C. Peng, G. H. Cohen, and R. J. Eisenberg. 1994. High-level expression and purification of secreted forms of herpes simplex virus type 1 glycoprotein gD synthesized by baculovirus-infected insect cells. *J. Virol.* 68:766-775.
 50. Spear, P. G. 1993. Membrane fusion induced by herpes simplex virus, p. 201-232. *In* J. Bents (ed.), *Viral fusion mechanisms*. CRC Press, Inc., Boca Raton, Fla.
 51. Stewart, P., and G. Nemerow. 1997. Recent structural solutions for antibody neutralization of viruses. *Trends Microbiol.* 5:229-233.
 52. Turner, A., B. Bruun, T. Minson, and H. Browne. 1998. Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. *J. Virol.* 72:873-875.
 53. Warner, M. S., W. Martinez, R. J. Geraghty, R. I. Montgomery, J. C. Whitbeck, R. Xu, R. J. Eisenberg, G. H. Cohen, and P. G. Spear. 1998. A cell surface protein with herpesvirus entry activity (HvE) confers susceptibility to infection by herpes simplex virus type 2, mutants of herpes simplex virus type 1 and pseudorabies virus. *Virology* 246:179-189.
 54. Whitbeck, J. C., C. Peng, H. Lou, R. Xu, S. H. Willis, M. Ponce de Leon, T. Peng, A. V. Nicola, R. I. Montgomery, M. S. Warner, A. M. Soulika, L. A. Spruce, W. T. Moore, J. D. Lambris, P. G. Spear, G. H. Cohen, and R. J. Eisenberg. 1997. Glycoprotein D of herpes simplex virus (HSV) binds directly to HVEM, a member of the TNFR superfamily and a mediator of HSV entry. *J. Virol.* 71:6083-6093.
 55. Whitbeck, J. C., C. Peng, G. H. Cohen, and R. J. Eisenberg. Unpublished data.
 56. Willis, S. H., C. Peng, M. Ponce de Leon, A. V. Nicola, A. H. Rux, G. H. Cohen, and R. J. Eisenberg. 1997. Expression and purification of secreted forms of herpes simplex virus glycoproteins from baculovirus-infected insect cells, p. 131-156. *In* M. S. Brown and A. R. MacLean (ed.), *Methods in molecular medicine: herpes simplex virus protocols*, vol. 10. Humana Press, Clifton, N.J.
 57. Willis, S. H., A. H. Rux, C. Peng, J. C. Whitbeck, A. V. Nicola, H. Lou, W. Hou, L. Salvador, G. H. Cohen, and R. J. Eisenberg. 1998. Examination of the kinetics of herpes simplex virus glycoprotein D binding to the herpesvirus entry mediator, using surface plasmon resonance. *J. Virol.* 72:5937-5947.
 58. Wittels, M., and P. G. Spear. 1990. Penetration of cells by herpes simplex virus does not require a low pH-dependent endocytic pathway. *Virus Res.* 18:271-290.
 59. WuDunn, D., and P. G. Spear. 1989. Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* 63:52-58.
 60. Zoller, M. J., and M. Smith. 1982. Oligonucleotide-directed mutagenesis using M13-derived vectors: an efficient and general procedure for the production of point mutations in any fragment of DNA. *Nucleic Acids Res.* 10:6487-6500.